Kisspeptin: a novel regulator of human foetal adrenocortical development and function

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Publications

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Dedication

To Jaja

Who always said, “You can do it.”

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Abstract

Context:

The human foetal adrenal (HFA) is an integral component of the foeto-placental unit and important for the maintenance of pregnancy. Low kisspeptin levels during pregnancy are associated with miscarriage, and kisspeptin and its receptor are expressed in the HFA. However, the role of kisspeptin in foetal adrenal function remains unknown.

Objective:

The objective of this work was to determine the role of kisspeptin signaling in the developing HFA.

Methods:

Experiments using H295R adrenocortical cells and primary HFA cells as *in vitro* models of the foetal adrenal were conducted. The spatiotemporal expression of the kisspeptin receptor, Kiss1R in the HFA was examined. The production of dehydroepiandrosterone sulphate (DHEAS) from HFA cells after kisspeptin treatment, alone or in combination with adrenocorticotropic hormone or corticotropin-releasing hormone was compared.

The association of plasma kisspeptin levels with HFA size was analysed in a longitudinal clinical study. Thirty-three healthy pregnant women were recruited at their 12-week routine antenatal ultrasound scan. Serial measurements of foetal adrenal volume (FAV) and maternal kisspeptin levels at four antenatal visits (~20, 28, 34, and 38 weeks' gestation) were collected. Outcomes of pregnancy were recorded, including neonatal auxology and complications in the postnatal period. Neoantal adrenal remodeling was characterized by

serial measurements of adrenal volume postnatally.

Results:

Expression of *Kiss1R* was present in the HFA from 8 weeks after conception to term and was shown in the inner foetal zone. Kisspeptin significantly increased DHEAS production in H295R and second-trimester HFA cells. Serial measurements of kisspeptin confirmed a correlation with FAV growth in the second trimester, independent of sex or estimated foetal weight.

Conclusions:

Together, these studies demonstrate that kisspeptin plays a key role in the regulation of the HFA, and thus the foeto-placental unit, particularly in the second trimester of pregnancy.

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List of Abbreviations

AC	abdominal circumference
ACTH	adrenocorticotropic hormone
AFP	alphafoetoprotein
AGP	adrenogonadal primordium
AHC	adrenal hypoplasia congenital
AngII	Angiotensin II
AP	adrenal primordium
ARC	arcuate nucleus
AS	antisense
AT1	angiotensin 1
AVPV	anteroventral periventricular nucleus
BPD	biparietal diameter
Brdu	5’-bromo-2’-deoxyuridine
BW	birth weight
BMI	body mass index

cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CRH	corticotropin-releasing hormone
Ct	cycle threshold
CYP	Cytochromes P450
DAX1	dosage-sensitive sex reversal, adrenal hypoplasia congenital (AHC), chromosome factor
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DZ	definitive zone
ECM	extracellular matrix
Edu	5-ethynyl-2'-deoxyuridine
Efw	estimated foetal weight
ELISA	enzyme-linked immunosorbent assay
Em LSCS	Emergency lower section caesarean section
FAdE	foetal adrenal-specific enhancer

FAV	foetal adrenal volume
FGF	fibroblast growth factor
FL	femur length
FZ	foetal zone
GA	gestational age
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC-MS	gas chromatography–mass spectrometry
Gli1	Glioma-Associated Oncogene Homolog 1
GnRH	gonadotropin-releasing hormone
GOI	gene of interest
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
hCG	Human chorionic gonadotropin
HDBR	Human Developmental Biology Resource
HDL	high density lipoprotein
HFA	human foetal adrenal

Hh	Hedgehog
ICH	Institute of Child Health
IGF	Insulin-like growth factor 2
IHH	idiopathic hypogonadotropic hypogonadism
IOL	induction of labour
kD	kilodalton
KISS1R	Kisspeptin receptor
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LDL	Low density lipoprotein
MC2R	melanocortin receptor type 2
MEC	meconium
mRNA	messenger RNA
MTA	Material transfer agreement
NNU	neonatal unit
NRES	National Research Ethics Service
NTC	no template control

PBS	phosphate buffer saline
PE	pre-eclampsia
PlGF	Placental growth factor
PKA	protein kinase A
PKC	protein kinase C
POMC	Pro-opiomelanocortin
PRA	plasma renin activity
PROM	prolonged rupture of membranes
PTCH1	Protein patched homolog 1 receptor
QMUL	Queen Mary University of London
qPCR	quantitative reverse transcriptase polymerase chain reaction
REC	Research ethics committee
RLH	Royal London Hospital
RNA	Ribonucleic acid
S	Sense
SD	standard deviation

SDS	standard deviation score
SER	smooth endoplasmic reticulum
SF1	steroidogenic factor 1
SHH	sonic hedgehog
SMO	Smoothened
SLOS	Smith Lemli Opitz syndrome
StAR	Steroidogenic acute regulatory protein
SULT2A1	sulphotransferase enzyme
SVD	spontaneous vaginal delivery
TZ	transitional zone
UCL	University College London
USS	ultrasound scan
Wpc	weeks postconception
ZG	zona glomerulosa
ZF	zona fasciculata

ZR	zona reticularis
3βHSD	3β hydroxysteroid dehydrogensase
17OHP	17 hydroxyprogesterone

1 INTRODUCTION

1.1 History of adrenal medicine

Despite their importance, anatomists neglected the adrenal glands for many years. Their discovery and description is generally attributed to the Italian anatomist Bartolomeo Eustachius in 1563. The uniqueness of the human foetal adrenal (HFA) was largely overlooked until 1911 when Stella Starkel and Leslaw Wagrzynowski, two Polish medical students, described the foetal zone (FZ) for the first time. The paper appeared in the German *Archiv für mikroskopische Anatomie und Entwicklungsgeschuchte,* and its original title was "Beitrag zur Histologie der Nebeniere bei Feten und Kindern" ("Contribution to histology of adrenals of foetuses and children"). The studies were performed on 100 adrenal glands obtained from foetuses (from 6 months of gestation) and children up to the age of five. They described the FZ as a "medullary zone", also as "immature cortex", which undergoes involution in first years of life. At the same time, Elliott and Armour published on “The development of the cortex in the human suprarenal gland and its condition in hemicephaly” also describing the unique FZ [1]. The transient FZ is not present in most mammals. It appears to be unique to humans and a few higher primates.

The true biological role of the adrenal gland only really became apparent when Addison described the effects of adrenal insufficiency in the middle of the 19th Century, and animal studies by Brown-Séquard reported the consequences of adrenalectomy. Harvey Cushing was the first physician to report the classic features of steroid hormone excess in 1932. During that decade it became clear that the adrenal gland produced two broad categories of steroids – termed “glucocorticoids” and “mineralocorticoids”. Subsequently, the laboratories of Reichstein and Kendall were successful in isolating and characterising the

structure of several key adrenal steroids, for which they jointly received the Nobel Prize for Medicine in 1950. More recently, advances in molecular genetics have helped to elucidate the pathological basis of many adrenal conditions.

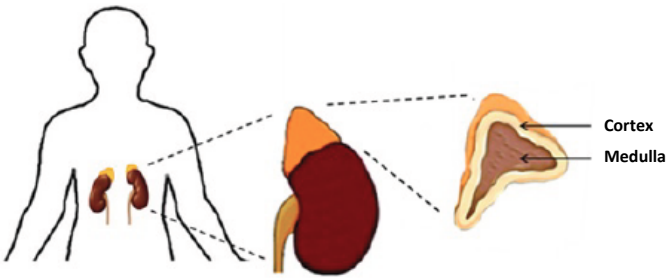
An appreciation of human adrenal development is essential to properly understand adrenal physiology, as well as the pathological basis of many congenital adrenal disorders. Furthermore, understanding the regulation of adrenal development will enlighten the emerging critical role the foetal adrenal plays as a component of the foeto-placental unit during pregnancy.

1.2 The human adrenal gland

1.2.1 Adrenal structure

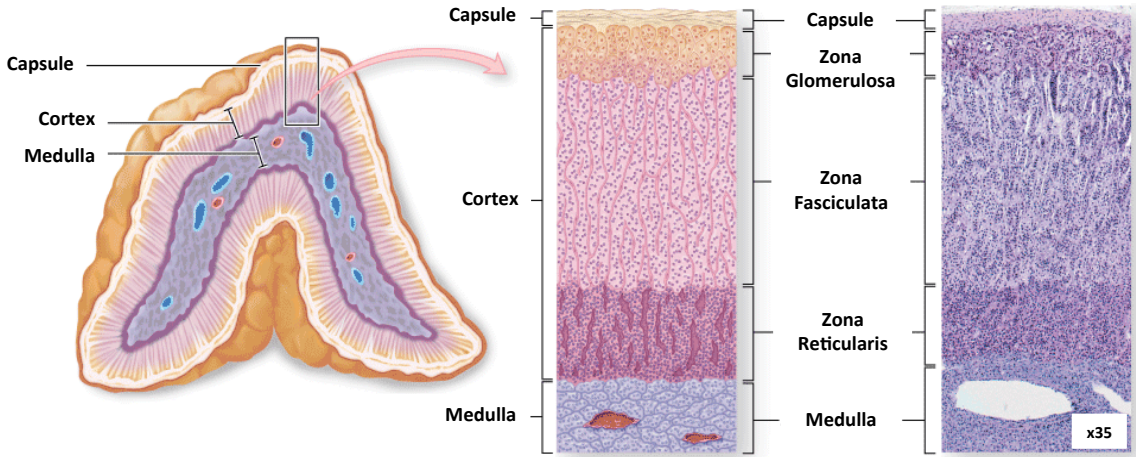
The adrenal glands are located anterior and superior to the upper pole of the kidney and comprised of an outer cortex and inner medulla (Fig. 1). These are embryonically distinct tissues, the cortex arising from the intermediate mesoderm, while the medulla is established from neuroectoderm. Adult human adrenals weigh 8-10 g and 90% is made up of the cortex. The medulla secretes catecholamines under conditions of stress that function as “fight or flight” hormones [2]. The adrenal cortex, unlike the medulla, is essential for life as it is the principal steroid producing organ in the body [3]. Adrenal failure or insufficiency can lead to disrupted electrolyte balance, impaired carbohydrate metabolism, and in the most severe cases, hypoglycaemic coma and death [4].

Fig. 1a. Anatomy and structure of the human adult adrenal gland.



The adrenals are located at the upper poles of the kidneys (Yates et al 2014).

Fig. 1b. The zones of the human adrenal cortex



The adult adrenal has three recognisable cortical zones, the zona glomerulosa (ZG), the zona fasciculata (ZF) and the zona reticularis (ZR) which surrounds the medulla [5]

Enclosing the adrenal gland is a capsule of fibroblasts and myofibroblasts (Fig. 1a), containing an arterial plexus, which sends trabeculae into the underlying cortex. Capsular cells are flattened with elongated nuclei arranged in the plane of the capsule. The cortex has three distinct concentric zones, named from outer to inner as the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR) [6]. Each zone can be distinguished easily on conventional staining by cell size and morphology. The narrow ZG, which makes up to 15% of the cortex contains small columnar epithelial cells (Fig. 1b) with basophilic cytoplasm and

low lipid content. Mineralocorticoids such as aldosterone are synthesised in the ZG and are responsible for regulating salt/water balance and blood volume [3,7]. Beneath the ZG is the ZF (Fig. 1b), which contains lipid-rich polygonal epithelial cells arranged in radial columns separated by fenestrated capillaries. The human ZG is arranged in discrete clusters such that some cords of ZF extend between them as far as the capsule [8,9]. The mitochondria of ZF cells have tubulovesicular cristae. Glucocorticoids such as cortisol (corticosterone in rodents) are secreted by the ZF [3], and have crucial effects on metabolism, the cardiovascular system and the immune system. The innermost zone is the ZR, comprised of polyhedral cells with prominent lysosomes (Fig. 1b). In humans and higher primates the ZR synthesises adrenal androgens such as dehydroepiandrosterone (DHEA) and its sulphate, dehydroepiandrosterone sulphate (DHEAS), also known as C_{19} steroids [3]. These are precursor sex hormones released into the blood stream, and taken up by the testis and ovaries to produce testosterone and oestrogen.

1.2.2 Adrenal Steroidogenesis

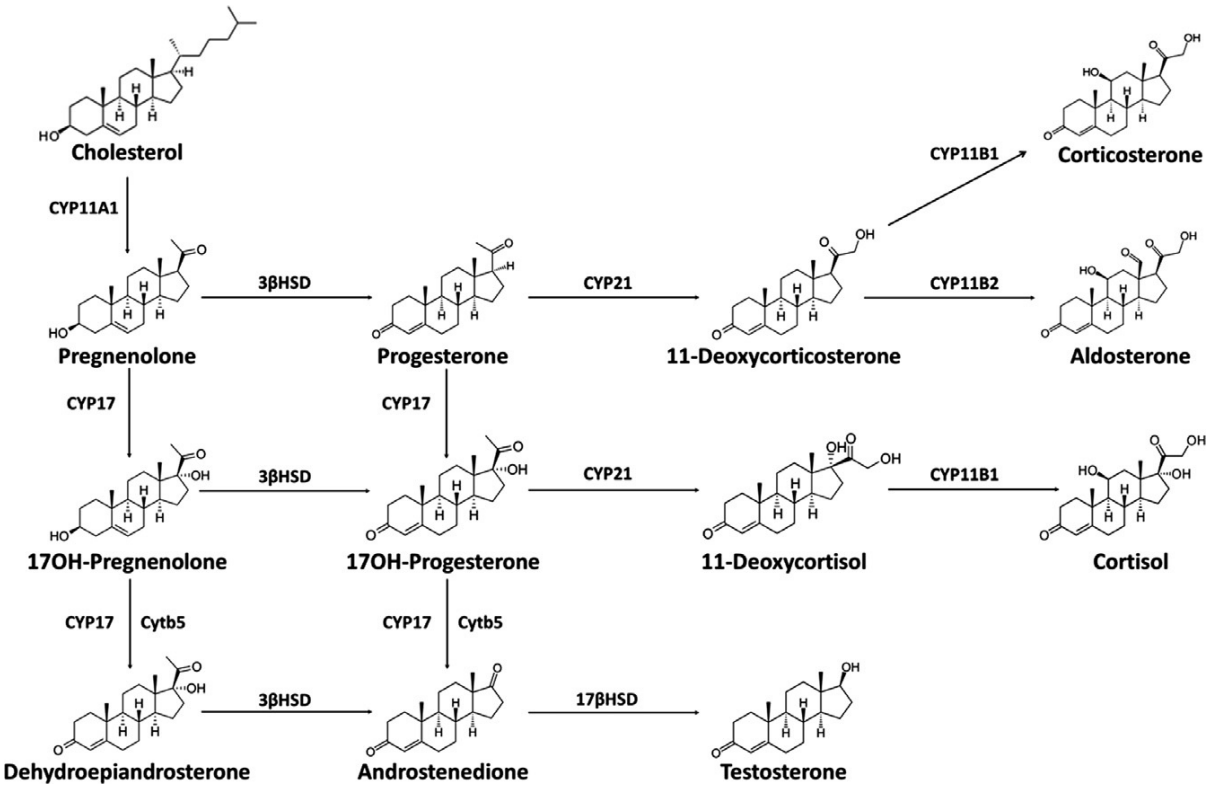
Steroidogenesis is dependant on the delivery of cholesterol to mitochondria and its catalysis by cytochrome P450 (CYP) mixed function oxidases and short-chain dehydrogenases. A series of reactions take place in the inner membrane of mitochondria or in the smooth endoplasmic reticulum (SER) with intermediate compounds shuttling back and forth [10]. The human adrenal cortex produces mineralocorticoids, glucocorticoids and androgens in the ZG, ZF and ZR respectively, the products being dependent on the zone-specific expression of steroidogenic enzymes and the stimulus.

The initial substrate cholesterol is synthesised *de novo* from acetate, or obtained from high-density lipoproteins (HDLs) internalised from the circulation by scavenger receptor B1, or low-density lipoproteins (LDLs) internalised by LDL receptor-mediated endocytosis [11]. Cholesterol is cleaved from its esterified form by hormone-sensitive lipase (HSL), previously known as cholesterol ester hydrolase.[12] Hormones, such as ACTH, stimulate the activity of this intracellular neutral lipase [13] and free cholesterol is then transported from the outer to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR). Following translocation, cholesterol is converted into steroids through sequential catalysis by a series of enzymes (Fig. 2). The first and only rate-limiting step involves the conversion of hydrophobic cholesterol to hydrophilic 21-carbon pregnenolone by P450 side chain cleavage (CYP11A1) enzyme. Pregnenolone is then translocated to the SER where, in humans, it is converted to 17OH pregnenolone by 17α-hydroxylase (CYP17) in the ZF and ZR, but not in the ZG where CYP17 is not expressed. 3β-hydroxysteroid dehydrogenase (3βHSD) converts 17OH pregnenolone to 17OH progesterone in the ZF and ZR, and pregnenolone to progesterone in the ZG. 21-hydroxylase (CYP21) converts progesterone and 17OH progesterone to 11-deoxycorticosterone and 11-deoxycortisol, respectively. These compounds are transported back to the inner mitochondrial membrane where the final steps of steroidogenesis take place. 11β-hydroxylase (CYP11B1) converts 11-deoxycortisol to cortisol in the ZF. In the ZG, 11-deoxycorticosterone is converted to aldosterone by aldosterone synthase (CYP11B2).

CYP17 acts as both a 17α-hydroxylase and a 17,20-lyase. Cytochrome b5, expressed in the ZR, promotes the lyase activity that cleaves two carbon atoms to produce DHEA from 17OH pregnenolone, such that in the ZR cortisol production is suppressed and adrenal androgens

form instead [14]. DHEA can be sulphated to DHEAS by the enzyme, sulphotransferase, SULT2A1, or converted to androstenedione by 3βHSD

Fig. 2. Adrenal steroidogenesis



The major steroid products secreted by the adrenal cortex, mineralocorticoids, glucocorticoids and adrenal androgens, are synthesised via a complex cascade of sequential reactions catalysed by steroidogenic enzymes (Yates et al 2014).

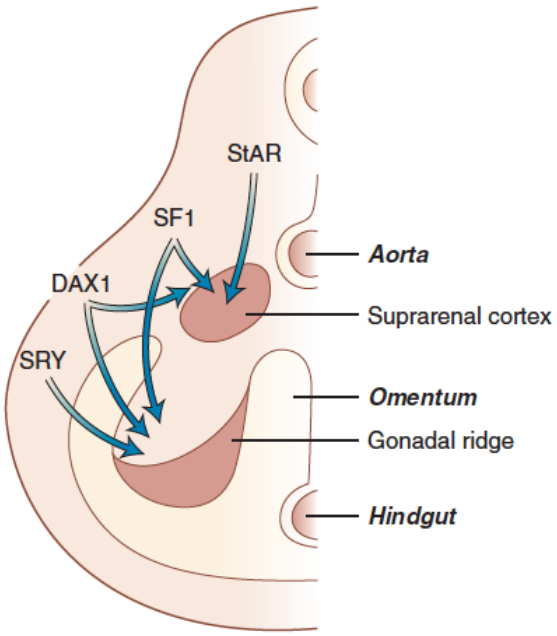
1.3 Human adrenal development

1.3.1 Embryology

The adrenal cortex develops from a thickening of the intermediate mesoderm known as the gonadal ridge, in contrast to the adrenal medulla, which derives from the ectoderm. The

adrenal gland appears as the bipotential adrenogonadal primordium (AGP) at 28-30 days post conception (dpc) in humans [15] due to expression of the transcription factor, steroidogenic factor 1 (SF1, NR5A1), a nuclear receptor essential for adrenal development and steroidogenesis (Fig. 3).

Fig. 3. Hemi-cross-section of a 5-week human embryo showing the locations of the adrenal primordia (suprarenal cortices) and gonadal ridges.



Steroidogenic factor 1 (*SF1*) is involved in testicular and ovarian development. *SRY* is the single critical regulator of testicular embryogenesis. Inactivation of the *DAX1* gene leads to adrenal hypoplasia. The steroidogenic acute regulatory protein (StAR) is the rate-limiting factor for adrenal steroidogenesis. [16].

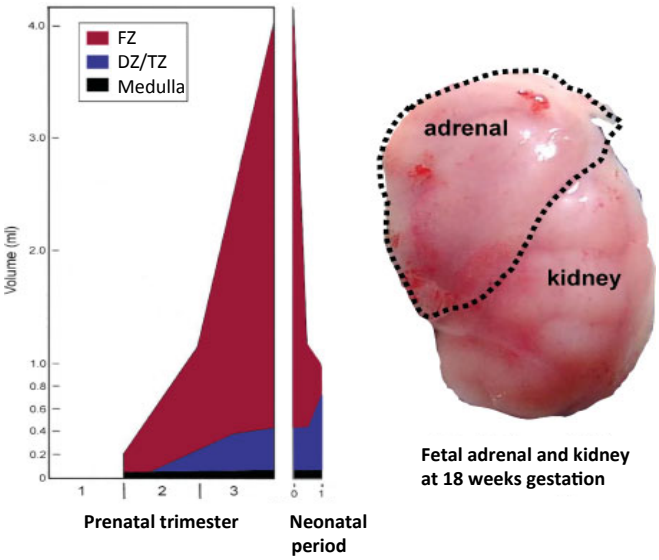
The gonadal cells migrate caudally. Those cells that are more medial, expressing the highest levels of SF1, migrate in a retroperitoneal fashion to the upper pole of the mesonephros to form the adrenal primordium at 33 dpc. At about 48 dpc sympathetic neural crest cells start migrating to the area where the adrenal primordium (AP) is developing. These cells persist

as clusters scattered throughout the foetal adrenal until birth, following which they coalesce and differentiate into the catecholamine-producing chromaffin cells of the adrenal medulla. Following the neural crest invasion, the adrenal primordium becomes encapsulated resulting in the formation of a distinct organ just above the developing kidney. By 50–52 dpc, within the cortex, there are two distinct zones. The inner zone, referred to as the foetal zone (FZ) contains large polyhedral, eosinophilic cells with high levels of expression of steroidogenic enzymes. The smaller outer zone, the definitive zone (DZ), is composed of small, tightly packed basophilic cells with much lower levels of steroidogenic enzyme expression. A third cortical zone, known as the transitional zone (TZ), becomes identifiable from around 14 weeks post conception (wpc). This TZ is located between the DZ and the FZ and contains cells whose histological appearance shares features of both[17].

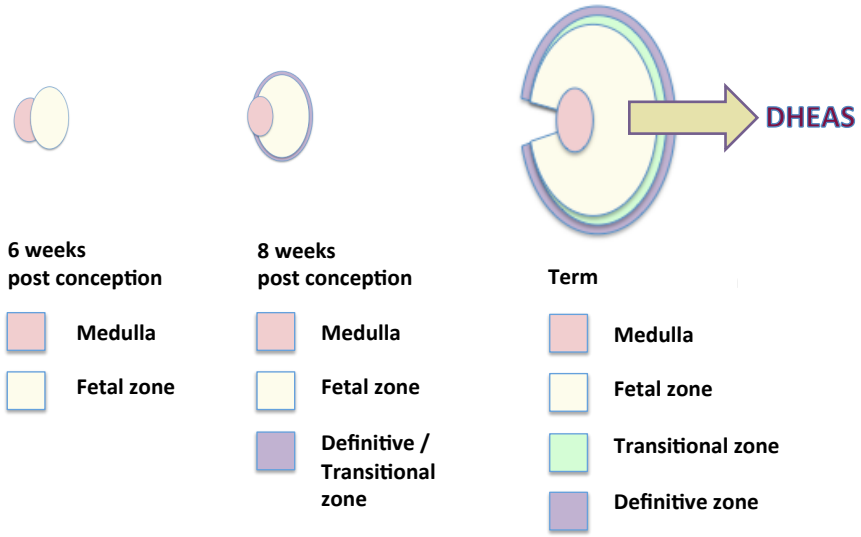
Following encapsulation the foetal adrenal grows rapidly, undergoing tremendous change as pregnancy progresses (Fig. 4a). This is largely due to an increase in size of the FZ, which accounts for 80-90% of the mass of the gland by mid-gestation [18,19]. Growth of the HFA is most rapid from the 2nd trimester and by 18 weeks the gland is approaching the size of the kidney (Fig. 4a). The fastest growth occurs during the last 6 weeks of gestation, mainly due to an enlarging FZ. The foetal adrenal forms 0.4% of body weight at term, weighing 3-5g with a relative size 10- to 20-fold that of the adult adrenal [20]. The main function of the foetal adrenal during pregnancy is steroidogenesis, specifically increasing C19 androgen production (Fig. 4b), and brief key periods of increased cortisol secretion. These steroids have crucial roles in maintaining intra-uterine homeostasis and in maturation of the foetus in preparation for extra-uterine life. FZ cells robustly express cytochrome P450 17α (CYP17), which converts pregnenolone to DHEA. DHEA is sulphated by the enzyme SULT2A1 to DHEAS, which is subsequently aromatised by the placenta to oestrogens. [20]. FZ cells also

produce other sulphated Δ5 steroids, including pregnenolone sulphate and 17α-hydroxypregnenolone sulphate but their functional role is unclear [15,20].

Fig. 4. Growth and functional development of the foetal adrenal



(4a) Size of the adrenal gland and its component parts in utero, during infancy, and during childhood. (Adapted from Bethune JE, The Adrenal Cortex: A Scope Monograph, Upjohn, 1974.) Photograph of an 18-week foetal kidney with adrenal [21]



(4b) Zonation of the foetal adrenal. As pregnancy progresses, the growth of the foetal adrenal is paralleled by increasing steroid production, principally DHEAS from the FZ (Adapted from Barwicka et al, 2005) [22]

1.3.2 Postnatal development

The HFA continues to undergo significant remodeling during neonatal and pubertal periods. Immediately after birth the FZ rapidly involutes and remodels by a process involving apoptosis of cells in the inner region of the FZ, and there is a concomitant decrease in adrenal androgen secretion [15]. The weight of the adrenal gland drops by 50% within the first 2 weeks after birth [23]. Whether the timing of foetal adrenal involution is determined by gestation or by birth is controversial. FZ androgen production has been reported to persist in infants born prematurely [24], and involution of the FZ may therefore be linked to maturation rather than the event of birth itself. In contrast, a more recent study has demonstrated a similar pattern of rapid adrenal involution, with size of the adrenal gland, as measured by ultrasonography, decreasing to its normal infantile size within the first 2 weeks after birth in all neonates examined, regardless of their gestational age at birth [20]. The FZ is absent by 6 months of age in most cases. Cells with ZR morphology are detectable in humans from around 3 years of age until a continuous ZR forms at around 6 years of age and adrenal androgen synthesis recommences, a stage referred to as adrenarche [25]. The exact mechanisms for ZR growth and the factors regulating adrenarche remain elusive. The adult cortex develops from the DZ and TZ, giving rise to the ZG and ZF, respectively.

1.3.3 Theories of growth and zonation of the adrenal cortex

It has long been thought that undifferentiated, pluripotent stem cells exist in the adrenal cortex to maintain homeostasis in the adult. The precise origin of adrenocortical cells has been debated over the years and their origin remains ambiguous. The adrenal cortex, as well as having a constant turnover of cells to replace dying cells, is a highly dynamic organ that undergoes rapid changes in response to hormonal demand and surgical injury,

following which it demonstrates remarkable regenerative capacity. A number of theories of the origin of the cells of the different adrenocortical zones have been proposed but the weight of evidence favours the centripetal migration hypothesis [20]. This postulates that there is a continual production of new cortical cells at the periphery of the gland that migrate under mitotic pressure to the inner cortical medulla boundary where they die [20]. Early lineage-tracing studies [26], transgenic studies [27,28], and genetic lineage tracing experiments [29,30] have provided direct evidence for the centripetal migration hypothesis.

Intravital dye-labeling experiments supported by observations from enucleation studies in rodents these cells originate in the capsule. Proliferation studies and lineage tracing experiments in mice propose sub capsular/DZ pools as the site of progenitor stem cells that differentiate as they move centripetally through the gland, with the implication that they change identity as they move between zones [29,30]. An alternative theory is that there is a population of stem cells in the foetal zone or transitional zone that can migrate in both directions to supply foetal and definitive zone cells. A combination of these concepts may be correct, with the foetal zone contributing to subscapular progenitor cells during development, that then differentiate through the various zones to maintain stable cell populations in the mature gland [31]. Some conditions associated with adrenal hypoplasia (for example, IMAGe syndrome, X-linked AHC) may reflect a defect in progenitor cell expansion during early foetal development.

Zubair, et al using the FAdE-Cre mouse model, observed proliferating cells in a scattered pattern throughout the adrenal gland until E13.5 At later time points, these cells were ordered in the periphery of the adrenal gland. Similar to the developing human adrenal cortex, the mouse FZ is first encapsulated by surrounding cells of the intermediate

mesoderm. Following encapsulation a second group of cells emerge as the densely packed DZ or definitive (adult) cortex. *Sf1* is activated by FAdE only in the FZ before E14.5 but this enhancer does not activate *Sf1* expression in the DZ. Zubair et al traced the fate of FZ cells during development and demonstrated that all DZ cells are derived from FAdE-expressing cells of the foetal adrenal. To complement these experiments, Zubair et al also utilized a tamoxifen-inducible FAdE-cre mouse model. DZ staining was only observed when tamoxifen was administrated early in embryogenesis (E11.5-E12.5). When tamoxifen was administered after E14.5, no LacZ-positive cells were found. These results are consistent with the absence of FAdE activity during later developmental stages and support the conclusion that the FZ gives rise to the DZ [32]. In contrast, studies focused on a downstream activator of the hedgehog pathway, Glioma-Associated Oncogene Homolog 1 (Zinc Finger Protein) (Gli1) provide evidence that the adrenal capsular cells also give rise to the DZ. Gli1-expressing cells are specifically located in the adrenal capsule and do not express Sf1. King et al demonstrated that this subpopulation of cells is capable of giving rise to Sf1-expressing, differentiated adrenocortical cells during embryonic development[30]. However, these data are in conflict with lineage tracing experiments using FAdE-LacZ reporter mouse that revealed that all DZ cells arise from FZ cells. These apparently conflicting observations may reflect two temporally distinct lineages of the definitive cortex. Wood et al demonstrated that FZ cells that once expressed *Sf1* under control of the FAdE enhancer gave rise to a subset of capsular cells. These FZ cell successors within the adrenal capsule express Gli1, suggesting some FZ cells can evolve into Sf1-negative capsular cells which in turn can give rise to the underlying DZ /adult cortex [33]. Recent lineage tracing studies have revealed that adrenal maintenance likely involves cell conversion of ZG cells into ZF cells [34], with cells being displaced in a centripetal fashion.

Several lines of evidence suggest that β-catenin plays an important role in adrenal zonation and maintenance. Activation of the β-catenin pathway is restricted to the ZG [35] and ectopic expression leads to the activation of ZG markers in ZF cells [36]. Vidal et al [37] identified members of the R-spondin (Rspo) gene family to be expressed from E12.5 onward within mesenchymal cells surrounding the forming adrenal. R-spondins are signaling molecules that positively regulate the β-catenin signaling pathway. This study showed capsular RSPO3 signals to the underlying steroidogenic compartment to induce β-catenin signaling and imprint glomerulosa cell fate. Shh in turn signals back to recruit capsular cells to form the adrenal cortex, at least during development. This study therefore identifies the adrenal capsule as a crucial signaling center that is continuously required for proper zonation.

1.3.4 Regulation of adrenal development

Our knowledge about the factors and underlying mechanisms that control adrenal development is limited at present. Based on human and animal models of disordered development and studies of gene expression during early human adrenal development, it is recognised that an array of transcription factors and signalling molecules are necessary for foetal adrenal growth and functional development.

1.3.4.1 Genetic regulation of adrenal development

The timing of gene expression is critical in the regulation of adrenal development. Genes involved in the formation of the intermediate mesoderm and urogenital ridge (Odd-skipped related 1 (*Odd1*), Wilms' tumor 1 (*WT1*), Sal-like 1 (Sall1), Pre-B-cell leukaemia transcription factor 1 (Pbx1), Wingless-type MMTV integration site family, member 4 (Wnt4), Forkhead box D1 and D2 (FoxD1 and FoxD2)) affect kidney, adrenal and gonadal development; those

affecting AGP development (steroidogenic factor 1 (SF1), dosage-sensitive sex reversal, adrenal hypoplasia congenital (AHC), X-chromosome factor (Dax-1), and Insulin-like growth factor receptor 1 (IGFR1)), affect both adrenal and gonadal development; there are also those that specifically affect adrenal development (CBP/p300-interacting transactivator with Glu/Asp-rich C-terminal domain, 2 (Cited2), Wnt/B-catenin signalling pathway genes) [31].

Of all the molecules regulating AGP development, the genes encoding SF1 (also known as NR5A1) and DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenital (AHC), X-chromosome factor) have emerged as pivotal. These genes show coordinated expression in adrenal cortex, testis, ovary, hypothalamus, and pituitary tissues [38]. In the absence of SF-1 expression, the adrenal gland does not form [15]. Severe disruption of SF-1 in humans can cause adrenal dysfunction, although must pathogenic variants in SF-1 in humans cause impaired testicular development and Leydig cell dysfunction rather than adrenal insufficiency. Sf1 haploinsufficiency (Sf1+/-) results in delayed/incomplete adrenal development in mice, while overexpression of Sf1 causes aberrant proliferation and neoplasia in the mouse adrenal. Several studies indicate that while Wilms tumor 1 (WT1) regulates *Sf1* expression in the AGP, CBP/P300-Interacting transactivator, with Glu/Asp-Rich carboxy-terminal domain, 2 (CITED2) expression in the AGP is necessary for proper differentiation of the adrenal primordial (AP or foetal zone- FZ) [15]. The foetal adrenal-specific enhancer, (FAdE) in the SF-1 locus that directs transgene expression to the foetal adrenal cortex was discussed above [32]. It was demonstrated that this enhancer is autoregulated by SF-1 and acts as a critical regulator of Sf1 gene expression in the mouse adrenal primordium. Sf1 subsequently regulates itself by maintaining FAdE-mediated *Sf1* expression in the AP. After E14.5 in mice, FAdE is no longer utilized. In the emerging DZ, *Sf1* regulation is shifted to a different definitive enhancer that has not yet been characterized.

In humans, however, no similar FADE or DZ enhancers have yet been confirmed [15].

SF1 upregulates Dax1, which in turn represses SF1 transcriptional activity and, hence, steroidogenesis. It is unclear how disruption of SF1 and its negative regulator can cause similar defects, but it may be that very fine control of SF1 activity is required for adrenal development. However, Dax1 can function as a co-activator for SF1 transcriptional activity in steroidogenic cells when expressed at high levels, and perhaps, the pleiotropic effects of these factors and not a simple consequence of their coactivity complicate the observations in vivo. Pathogenic variants or deletions of *DAX1* in humans are well established as the cause of X-linked adrenal hypoplasia congenita (AHC) [39]. Knockdown of *Dax1* results in premature differentiation of mouse adrenocortical progenitor cells. However, this occurs at the expense of depleting this essential cell population, ultimately resulting in adrenal failure. As such, Dax1 plays an essential role in the maintenance of stem/progenitor cell pluripotency. Sf1 activates *Dax1* transcription in cooperation with paracrine Wnt signalling and glucocorticoids that are synthesized in the differentiated adult cortex. Conversely, ACTH, the well-established glucocorticoid stimulator, has been shown to effect release of Sf1 complexes from the *Dax1* promoter, thus leading to effective inhibition of *Dax1* transcription [40,41]. This is predicted to promote the response of Sf1-positive progenitor cells to ACTH and subsequently initiate steroidogenesis.

1.3.4.2 Signalling pathways involved in adrenal development

Sonic hedgehog (Shh) is a member of the vertebrate hedgehog (Hh) family of secreted ligands, and performs a multitude of crucial roles during embryonic development and is also required in the adult for tissue maintenance, differentiation, and the regulation of stem cell populations. Secreted Hh ligands bind to the 12-pass transmembrane protein Patched-1 (Ptch1), which relieves the inhibition it exerts on the G protein-coupled receptor smoothened (Smo) and allows it to prevent the processing of the Gli transcription factors. Full-length Gli3 and Gli2 act as transcriptional activators. Gli1, which only acts as a transcriptional activator, is not expressed in the absence of Hh, but is upregulated by the pathway. Shh signalling is required for normal adrenal development at a later stage than SF1 and DAX1, and is expressed in the adrenal primordium in cells just beneath the capsule [29,42,43]. Shh expression marks cortical progenitors [42] and Shh expressing cells give rise to all steroidogenic cells in cortical zones. Homozygous deletion of Shh in mice is embryonically lethal. Analysis of the adrenal gland at 14.5 and 16.5 dpc in Shh-/- embryos, however, indicates that the adrenal primordium forms but is much smaller than in the wild type [29,43].

Holoprosencephaly is a consequence of the inactivation of the Hh pathway, with defects observed in SHH [44], PTCH1, and GLI2, and is often associated with adrenal hypoplasia [45]. Smith–Lemli–Opitz syndrome (SLOS), a condition with defects in 7-dehydrocholesterol reductase, (required for the formation of cholesterol), is associated with abnormalities that overlap with those seen in cases of impaired Hh signalling, including holoprosencephaly [46]. SLOS is also associated with adrenal insufficiency [47]. This may be due to the requirement of cholesterol as the substrate for steroidogenesis, or may be linked to two observations regarding Hh signalling. First, Hhs are covalently linked to cholesterol [48], the only such

proteins known to undergo this modification, which is critical for their signalling and second, 7-dehydrocholesterol, which accumulates in the plasma of SLOS patients can be transported out of the cell by Ptch1 and act as a negative regulator of Smo [49].

Fibroblast growth factor (FGF) signalling controls early developmental processes such as cell movement during gastrulation, mesodermal and neuroectoderm formation, anterior/posterior patterning, and organogenesis [50]. FGFs are a large family of secreted glycoproteins that bind to four signalling FGF receptors, FGFR1–4. FGF signalling interacts with SF1 and Shh signalling [51]. *Fgfr2* and *Fgfr4* are expressed in the developing adrenal cortex. Embryos with a global Fgfr2 IIIb deletion have hypoplastic adrenal glands [51], and deletion of both isoforms of FGFR2 from steroidogenic tissue recapitulates this phenotype and causes male-to-female sex reversal, implying that FGFR2 is not necessary for AGP formation but is required for the subsequent growth and development of the adrenal gland [52].

Epidermal growth factor (EGF) stimulates proliferation of both the foetal and definitive zones. The foetal adrenal expresses high levels of IGF-2 mRNA and protein, which are responsive to ACTH [53]. IGF-2 augments ACTH-stimulated expression of steroidogenic enzymes and stimulates steroid hormone production in foetal adrenal cortical cells. The pattern of enzyme maturation in the foetal adrenal suggests that cortisol production by the definitive zone does not occur de novo from cholesterol until 30 weeks of gestation, but some production using progesterone as precursor probably occurs earlier [53].

CDKN1C (Cyclin Dependent Kinase inhibitor 1 C, P57KIP2) is a paternally imprinted gene, located on chromosome 11p.15, and encoding for the CDKN1C protein, an inhibitor of cell

cycle progression. Variations in CDKN1C or its genomic imprinting can lead to adrenal pathology [54]. Loss-of-function of CDKN1C results in Beckwith-Wiedemann syndrome, an overgrowth syndrome with increased susceptibility to adrenal carcinoma. IMAGe (intrauterine growth restriction, metaphyseal dysplasia, AHC, and genital anomalies) syndrome is a rare multisystem disorder [54] that mirrors the features of Beckwith Wiedemann syndrome and is caused by gain-of-function mutations in CDKN1C. Most affected individuals are born small and develop skeletal abnormalities. Males present with genital malformations including micropenis and undescended testicles and urethra anomalies, but the most clinically important condition of the syndrome is adrenal insufficiency, which shortly after birth causes salt wasting, hypoglycemia, and shock due to loss of both mineralocorticoid and glucocorticoid synthesis and can be life-threatening [54].

1.3.5 Models to study adrenal development

A number of *in vitro* cell models are available to study adrenal development. These differ in terms of capacity for cell growth, response to agonists and steroidogenic capacity. Each may also have changing properties in culture over time.

The human adrenal carcinoma cell line NCI-H295 was isolated from an adrenocortical carcinoma of a 48-year-old Afro-Caribbean female patient [55]. This was the first adrenal cell line to express all enzymes required for steroidogenesis and to retain steroidogenic capacity [56]. H295 cells are aneuploid and hypertriploid, with the modal number of chromosomes being 62, held by approximately 30% of cells. This original line grows slowly in suspension, with a doubling time of approximately 5 days [57]. They produce their own cholesterol for steroidogenesis, and like the tumour from which they originated, mainly produce

androgens, with low mineralocorticoid and glucocorticoid synthesis, making them analogous to the foetal adrenal, with pluripotent capabilities [55]. Like adrenocortical cells *in vivo*, they respond to second messenger pathways [56]. AngII increases intracellular calcium levels via AT1 activation, and this along with PKC signalling preferentially induces CYP11B2 transcription to produce aldosterone [58]. Potassium ions also cause increased intracellular calcium levels, and aldosterone release [59]. This response is identical to AngII and potassium ion stimulation of the ZG. However, this strain demonstrates minimal response to stimulation with ACTH, which may in part be due to low expression levels of the ACTH receptor, MC2R [60]. The cAMP (cyclic adenosine monophosphate) pathway can be activated by forskolin, which acts at adenylate cyclase and also activates the protein kinase A (PKA) pathway. This results transcription of CYP17 and CYP11B1 and hence production of cortisol [61]. Chronic treatment with forskolin promotes production of androgens [62].

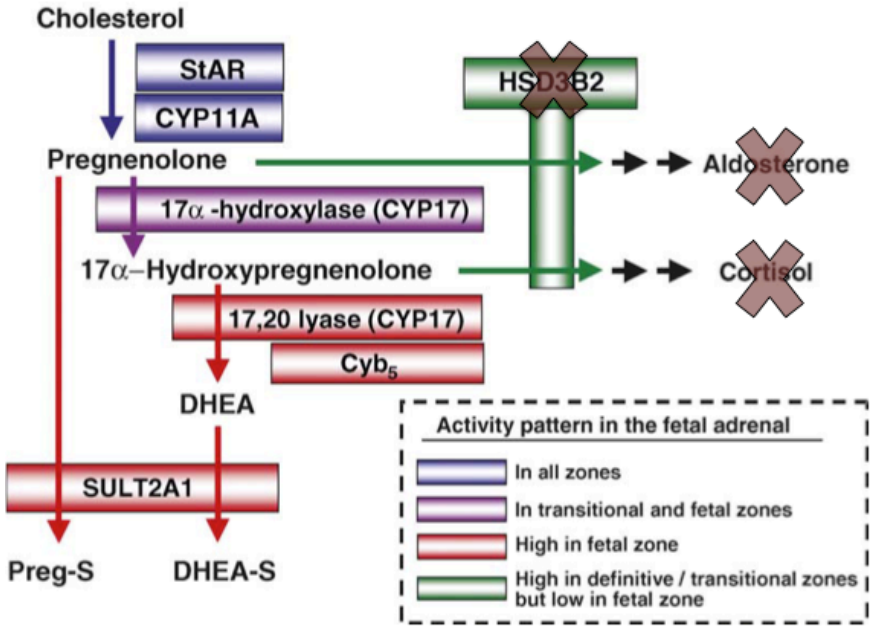
A strain of H295 cells, named H295R, was selected for their shorter population doubling time of ~2 days (the original line grows slowly in suspension with a doubling time of ~5 days), and their ability to grow in an adherent monolayer [57]. Both strains can be grown in 50% (v/v) Dulbecco's Modified Eagles Medium, 50% (v/v) Nutrient Mixture F-12 Ham, 2% (v/v) Ultroser G (a bovine derived serum substitute to increase growth rate and help retain steroidogenic capacity), 1% (v/v) ITS containing 1mg/ml insulin, 0.55mg/ml transferrin, and 0.5µg/ml sodium selenite, and 1% (w/v) Pen/Strep (5000U/ml penicillin and 5mg/ml streptomycin). H295R cells produce mainly androgens with low glucocorticoid and mineralocorticoid production, making them analogous to the foetal adrenal with pluripotent capacity [55]. Therefore this cell line, established in our laboratory, was selected for the preliminary studies described later.

Alternatives include the use of animal models or primary cultures of HFA cells. The developing human adrenal contains the unique inner FZ. Rodent models have been used to study adrenal physiology and pathology. The murine foetal adrenal contains the inner X zone, which has been proposed as analogous to the FZ of the human, although there is strong evidence that this is not the case [63]. The X zone persists until puberty in males, and the first pregnancy in females, in contrast to the FZ, which involutes rapidly after birth in the neonatal period. The X zone is of unknown function and crucially does not produce androgens.

1.3.6 Foetal adrenal steroidogenesis

Steroid production from the foetal adrenal has important roles in the maintenance of intrauterine homeostasis and in the maturation of the foetus in preparation for postnatal adaptation to extrauterine life.

Fig. 5. Steroidogenic enzyme expression in the human foetal adrenal zones (adapted from [21]).

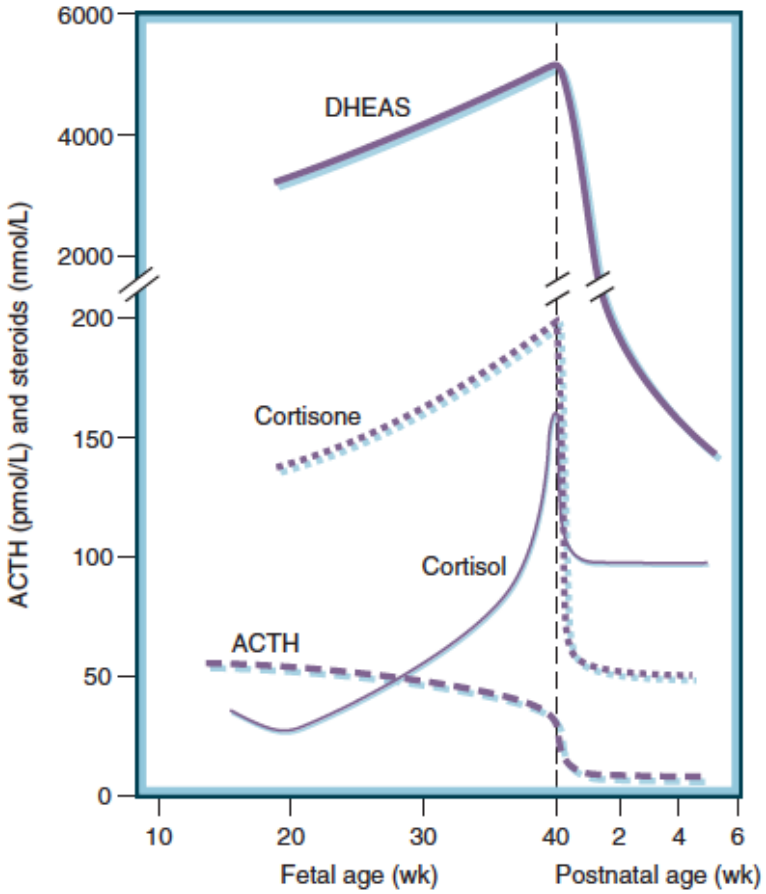


Transcription of steroidogenic genes is tightly regulated resulting in differences in the zonal activity of these enzymes [20]. During most of gestation, the foetal adrenal lacks HSD3B2, preventing cortisol and aldosterone synthesis and directing steroid production toward DHEAS production. DHEAS biosynthesis requires only three enzymes, localised in the mitochondria (CYP11A), endoplasmic reticulum (CYP17, 17α-hydroxylase/17-20-lyase), and cytosol (SULT2A1). Levels of these enzymes are very high in the HFA and vary in their expression within each adrenal zone[20].

The principal output from the foetal adrenal during pregnancy is DHEA from the FZ, which is then sulphated to dehydroepiandrosterone DHEAS by the enzyme sulphotransferase (SULT2A1). The DZ/TZ contributes only a small fraction of total foetal adrenal steroid production. Studies using immunohistochemistry have defined zonal differences in foetal adrenal expression of steroidogenic enzymes (Fig. 5).

The TZ produces cortisol, with an early peak from 8-9 wpc coinciding with transient expression of type 2 3β-hydroxysteroid dehydrogenase (HSD3B2) [64]. HSD3B2 is the enzyme needed for the conversion of Δ5 to Δ4 steroids (specifically pregnenolone and 17**α**-hydroxypregnenolone to progesterone and 17**α**-hydroxyprogesterone, respectively). The hypothalamic-pituitary-adrenal axis is sensitive to glucocorticoid-mediated feedback at this time; 46,XX foetuses with steroidogenic defects (e.g., in CYP21 or CYP11) lack cortisol and have an elevated ACTH drive that results in excess production of foetal androgens at a time when the genital and scrotal folds are sensitive to androgen exposure, resulting in virilization of female genitalia [64]. Cortisol can retard foetal and placental growth and so is converted to biologically inactive cortisone by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) in placental and foetal tissues. In this way the foetus is protected from high levels of cortisol *in utero*. Levels of circulating cortisone in the foetus at mid-gestation are 4-5 fold higher than cortisol concentrations (Fig. 6).

Fig. 6. Patterns of change of foetal plasma adrenocorticotropic hormone (ACTH), cortisol, cortisone, and dehydroepiandrosterone sulphate (DHEAS) during gestation and in the neonatal period.



The trend of average values is shown for each hormone in nanomoles per liter. Note the broken scale for DHEAS [16]

As term approaches, expression/activity of 11βHSD2 drops and selected foetal tissues including liver and lung express 11βHSD1, so that maternal glucocorticoids are available to the foetus to promote the maturation of foetal organs, including the lung and brain. This increase in foetal cortisol production has an important role for extra-uterine survival. Mice lacking glucocorticoid receptor function manifest enlarged and disorganized adrenal

cortices, adrenal medullary atrophy, lung hypoplasia, and defective gluconeogenesis [15] They appear normal at birth but are not viable.

The DZ expresses the enzymes needed for the production of aldosterone but does not express 17-hydroxylase or SULT2A1. The HFA is capable of aldosterone secretion near term, and foetal plasma aldosterone concentrations in infants who are born by caesarean section are 3- or 4-fold higher than maternal levels [6565]. Vaginal delivery and maternal salt restriction increase concentrations in both mother and infant. The increased aldosterone concentrations in the foetus are a result of increased foetal adrenal secretion and persist during the first year of extrauterine life. However, there is a poor correlation between plasma renin activity (PRA) and aldosterone concentrations in cord blood [66].

During most of gestation, the foetal adrenal lacks HSD3B2, preventing cortisol and aldosterone synthesis and directing steroid production toward DHEAS production. High expression of SULT2A1 in the FZ accounts for sulphation of most of the Δ5 steroids produced, including DHEA and pregnenolone. DHEAS biosynthesis requires only three enzymes, localised in the mitochondria (CYP11A), endoplasmic reticulum (CYP17), and cytosol (SULT2A1). Levels of these enzymes are very high in the HFA and vary in their expression within each adrenal zone. Large amounts of cholesterol are required as a precursor for steroid production and they are obtained through low-density lipoprotein receptors on the cell surface as well as intracellular synthesis of cholesterol from acetate. As in the adult adrenal, cholesterol use for steroids is tightly controlled by the expression of *StAR*, which regulates transport of cholesterol to the inner mitochondrial membrane, where CYP11A converts it to pregnenolone. DHEA is sulphated in the FZ to DHEAS, which is then converted by placental aromatase to oestrogens.

1.4 The foeto-placental unit

Successful pregnancy entails the timely birth of an appropriately mature foetus. Complex genetic, cellular, and hormonal interactions are necessary to facilitate implantation, placentation, embryonic development and foetal maturation, parturition and transition to extra-uterine life. Human pregnancy involves an orchestra of autocrine, paracrine and endocrine networks that coordinate maternal-placental-foetal communication. The foetal endocrine milieu is largely independent of maternal hormones because the placenta is impermeable to most peptide hormones. Hormones larger than 0.7-1.2 kD have little or no access to the foetal compartment [67]. Unique features of the foeto-placental endocrine environment include a growing spectrum of placental hormones and growth factors and a variety of foetal endocrine adaptations to the intrauterine environment, and the foetal adrenal cortex is prominent among these.

Oestrogens modulate many intrauterine processes throughout gestation, and the balance between oestrogens and progesterone *in utero* is thought to be critical to the maintenance of pregnancy, foetal maturation and the onset of parturition [68]. It is important to note that early increased exposure to progesterone can advance the endometrium, resulting in asynchrony of embryo development to endometrial development and reduction of implantation [69,70]. Generally, however, progesterone maintains pregnancy by promoting uterine quiescence. Any disruption of progesterone synthesis or action during pregnancy rapidly induces abortion or delivery [71]. In contrast, oestrogens oppose this and promote myometrial contractility of the uterus and regulate cervical ripening to facilitate parturition.

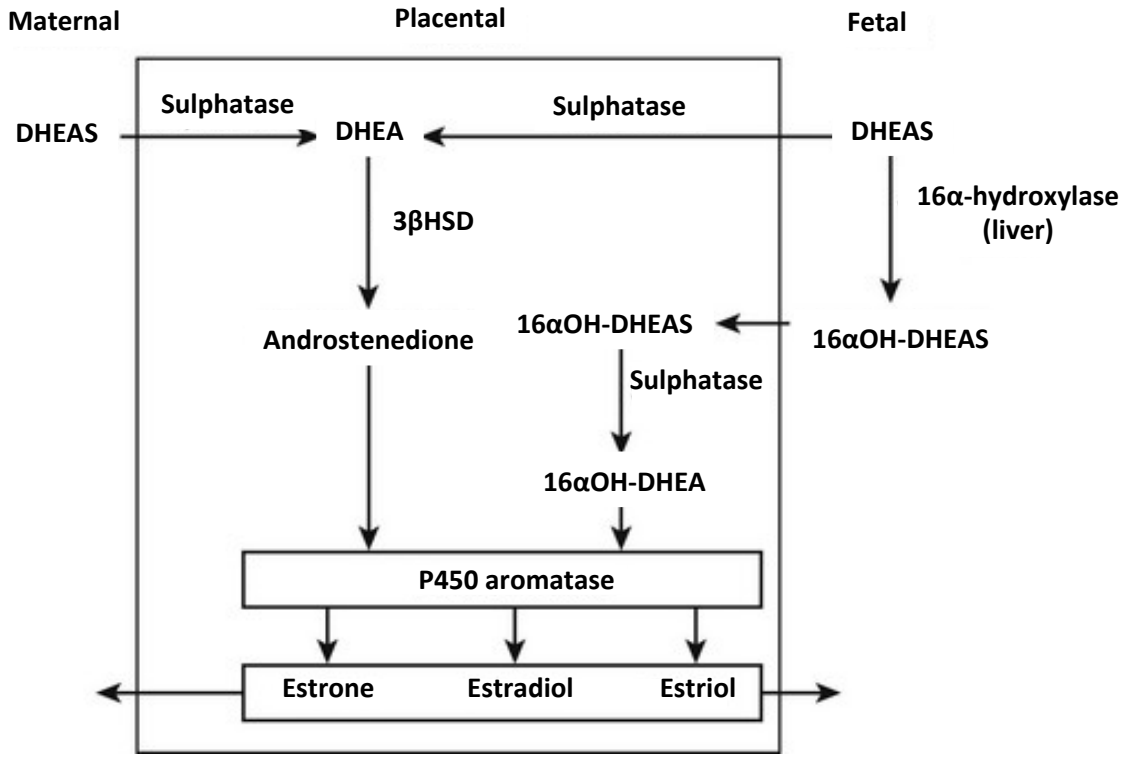
The placenta produces vast amounts of oestrogens in the form of oestradiol, oestrone, oestriol and oestetrol. The oestrogen products released from the placenta depend on the

nature of the substrate available. Oestradiol is the primary oestrogen circulating at term. In addition, however, significant levels of oestriol and oestetrol are also found in the maternal circulation and they increase particularly late in gestation. These hydroxylated forms of oestrogen are produced in the placenta using substrates from the combined efforts of the foetal adrenal gland and liver. Though the primary site of oestrogen biosynthesis is the placenta, the placenta lacks the cytochrome P450 enzyme CYP17 and accordingly is unable to synthesise oestrogens *de novo* [20]. Placental oestrogen biosynthesis relies on a supply of C_{19} androgens, mainly DHEA and its sulpho-conjugate, DHEAS, derived principally from the foetal and maternal adrenal cortex [20,53,68]. The foetal adrenal cortex contributes a greater proportion of substrates for placental oestrogen synthesis [68]. It has long been known that women pregnant with an anencephalic foetus have much lower levels of circulating oestrogens [72,73]. Pointing to the characteristic absence of the FZ of the adrenal cortex in anencephaly, Frandsen and Stakeman [72] postulated that the normal foetal adrenal produces a substance that serves as a precursor for oestrogen biosynthesis by the placenta. This, together with the finding of high levels of DHEAS in cord blood of normal newborns, suggested that foetal adrenal cortex is the principal source of placental oestrogen precursors. Confirmation was provided by Diczfalusy's group who demonstrated that radiolabeled DHEAS that had perfused through the placenta was converted to oestradiol [74,75]

In the placenta the sulphate moiety is removed from DHEAS by the sulphatase enzyme, and DHEA is then converted to androstenedione and testosterone by the actions of 3β- and 17β-hydroxysteroid dehydrogenase (3β- and 17β-HSD) enzymes, which are subsequently aromatised respectively to oestrone and oestradiol (Fig. 6). Placental 17β-HSD is considered to prevent passage of excessive oestrogens to the foetus by catalyzing inactivation of

oestradiol to oestrone [76]. Oestriol production increases as pregnancy progresses and exceeds the production rates of oestrone and oestradiol by late gestation [20]. The placenta lacks the 16α-hydroxylase enzyme and can only produce oestriol from a 16α-hydroxylated C19 precursor (Fig. 7). 16α-hydroxy DHEAS is produced principally by the foetal liver (and to a limited extent by the foetal adrenal). Placental oestriol production therefore reflects foetal adrenal steroidogenic activity. Oestetrol is produced after 15α- and 16α-hydroxylation by the foetal liver but placental production of this oestrogen is limited and its roles unknown.

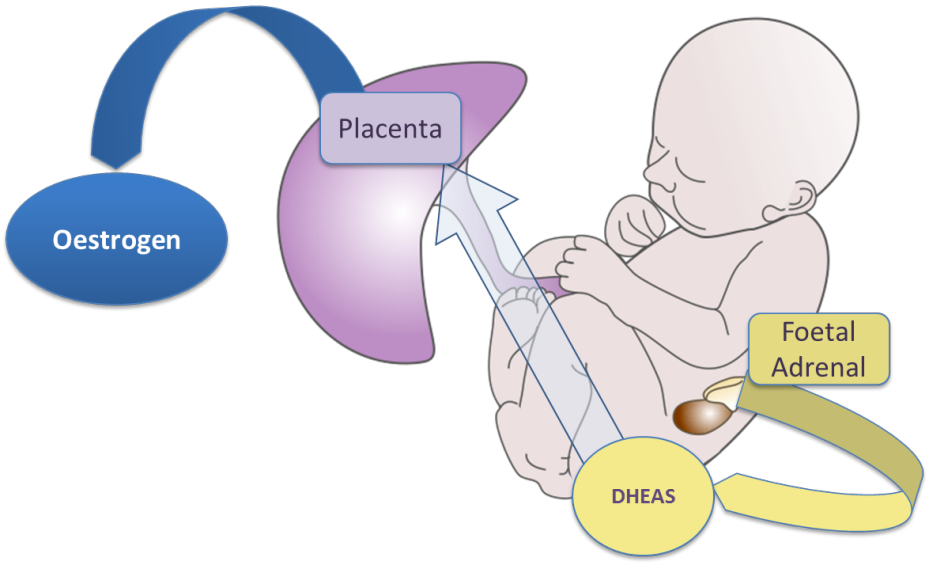
Fig. 7. Pathways for the biosynthesis of placental oestrogens



At term nearly 60% of the total DHEA used by the placenta for oestradiol and oestrone synthesis is derived from the foetal adrenal, whereas 40% is derived from the maternal adrenal [20,21,68,77]. Furthermore, 90% of 16 α-hydroxyl DHEAS, used as a substrate for oestriol synthesis, is foetally derived [20,68]. In this way the foetal adrenal and the placenta function

together as a foeto-placental unit (Fig. 8). Hence appropriate development and function of the foetal adrenal is critical for the maintenance of a pregnancy, enabling foetal maturation to ensure future perinatal survival.

Fig. 8. Foeto-placental unit.



The foetal adrenal is almost solely dedicated during the majority of gestation to the production of dehydroepiandrosterone sulphate, DHEAS. This C19 androgen is aromatised in the placenta enabling the production of oestrogens.

HFA function is programmed by the steroidogenic enzyme expression pattern to produce DHEA and pregnenolone and their sulphate conjugates. Much of the DHEA is converted to 16-hydroxy-DHEAS by the foetal adrenal and foetal liver. This programming is designed to provide DHEA substrate for placental oestrone and oestradiol production: 16-hydroxy-DHEA undergoes metabolism to oestriol in the placenta. Foetal DHEAS production and maternal oestriol concentrations increase progressively to term; DHEAS production approximates to

200 mg/day near term [78]. In pregnant baboons with placental oestrogen production suppressed by administration of an aromatase inhibitor, the volume of the foetal zone of the foetal adrenal increased markedly [79]. This effect was reversed by administration of inhibitor plus oestrogen, suggesting that oestrogen selectively suppresses foetal zone growth and development during the second half of primate pregnancy. It is proposed that this represents a feedback system to regulate secretion of foetal adrenal DHEA, thereby maintaining normal foetal-placental function and development [79]

1.5 Hormonal regulation of adrenal development

1.5.1 ACTH and the foetal hypothalamic-pituitary-adrenal axis

A major stimulus to foetal adrenal function is foetal pituitary ACTH. ACTH is a 39 amino acid peptide secreted from the anterior pituitary gland under control of corticotropin-releasing hormone (CRH) [80]. CRH neurons projecting from the paraventricular nucleus onto proopiomelanocortin (POMC)-containing neurons in the hypothalamic arcuate nucleus can also stimulate POMC release which is cleaved to form ACTH [81]. ACTH binds to the transmembrane receptor melanocortin receptor 2 (MC2R) specifically in adrenocortical cells. MC2R is a 7-transmembrane G protein-coupled receptor (GPCR), and binding of ACTH causes the α–subunit of the stimulatory heterotrimeric G protein (Gαs) to associate with adenylate cyclase. This generates cyclic adenosine monophosphate (cAMP), leading to the activation of downstream signalling pathways including activation of protein kinase A (PKA). PKA phosphorylates and activates cholesterol ester hydrolases and StAR, increasing the amount of cholesterol delivered to the inner mitochondrial membrane. cAMP also induces transcription of StAR, CYP11A1 and CYP17 to promote cortisol production [82,83]. In a typical

negative feedback loop, glucocorticoids inhibit further ACTH secretion at both the hypothalamic and pituitary level [84].

ACTH has been prposed to be the most potent stimulus of the adrenal cortex, and most of the knowledge on its mechanism of action derives from studies on the adrenal cortex or ACTH receptor-expressing cells [85]. However several observations suggest ACTH is not the sole factor that stimulates adrenal growth. ACTH is involved in various aspects of the dynamic organization of the adrenal cortex, namely cell migration and proliferation. While ACTH has been shown to stimulate proliferation of cells *in vivo*, it is not a mitogen *in vitro*. It has been shown that ACTH clearly inhibits the growth of adrenal cells *in vitro*[86] and furthermore, treatment of rats with antiserum raised against ACTH, although decreasing serum corticosteroid concentration, does not result in adrenal atrophy [87]. The trophic actions of ACTH may therefore be mediated by locally produced growth factors, such as insulin-like growth factor 2 (IGF2), fibroblast growth factor beta (βFGF, FGF2) [15] and epidermal growth factor (EGF), that act in an autocrine and/or paracrine fashion [20]. ACTH results from proconvertase (PC) PC1/3 cleavage of the proopiomelanocortin (POMC) precursor and may be further cleaved by proconvertase 2 to generate α-melanocyte-stimulating hormone (α-MSH) (amino acids 1–13 of ACTH) [88]. There is mounting evidence that proliferation is more likely to be mediated by other POMC-related peptides.

The structure of POMC was revealed in the late 1970s and the discovery that the precursor contained other, previously uncharacterized, peptides resulted in further lines of work including elucidation of the role of the N-terminal 16K fragment or pro-γ-MSH [89]. In humans, the 16K fragment is a 76-residue peptide with the sequence of γ3-MSH at its C-terminal, and the peptide is referred to as pro-γ-MSH (or N-POMC(1–76)) [89]. Further investigations have

shown that the active domain of POMC-derived peptide is a small fragment, N-POMC (50–74) (also named γ3-melanocyte-stimulating hormone, γ3-MSH) [89]. Several studies have demonstrated that this peptide is one of the principal processed products secreted from the pituitary corticotroph cell into the circulation [90,91], however though evidence strongly points to a role for pro-γ-MSH in the regulation of adrenal growth, the underlying mechanisms remain unclear [89].

Hypophysectomy of experimental animals results in adrenocortical atrophy, while excess ACTH states such as Cushing's disease cause adrenocortical hyperplasia [20]. The *Pomc* null mouse, in a similar way to hypophysectomised animals, could provide a model to investigate the role of different POMC peptides in adrenal function. *Pomc*-null mice are born with normal adrenal glands [92] but soon after birth the gland atrophies, indicating the requirement for POMC peptides for adrenal maintenance and growth [93]. Studies of the adrenals in Pomc null mice [94] have shown that the adrenals subsequently underwent atrophy after birth due to a lack of cellular proliferation rather than apoptosis. It has been reported that when *Pomc* null mice were injected on a daily basis for a period of 10 days with a high dose of ACTH(1–24) (around 10,000 times basal levels), an increase in adrenal size and also restoration of corticosterone but not aldosterone levels was seen [95]. However, examination of the adrenals showed that the increase in size was a result of cellular hypertrophy with no evidence of any hyperplasia. Stimulation of the adrenals in young *Pomc* null mice with lower doses of ACTH did not result in secretion of corticosterone, although the adrenals at this stage appeared relatively normal, expressing both the ACTH receptor and components of the steroidogenic pathway [94]. Most significantly, the authors transplanted single adrenals from 9-day-old *Pomc* null mice into bilateral adrenalectomised wild-type littermates, exposing the mutant adrenals to physiological levels of all of the

POMC peptides. After 3 months, it was noted the transplanted adrenal appeared histologically normal and fully functional producing normal levels of both aldosterone and corticosterone. This result demonstrates that when exposed to all the POMC peptides, the mutant adrenals can become fully functional glands [94]. In a reconstitution experiment, Pomc null mice were injected with synthetic N-POMC(1–28) (twice daily) or supraphysiological concentrations of ACTH(1–24) (daily) either alone or in combination [93]. Treatment with ACTH alone again resulted in adrenal cellular hypertrophy with no cell proliferation, as shown previously [95]. However, treatment with N-POMC(1–28) alone had no apparent effect on the adrenals. When both peptides were administered together, the adrenals were identical to when treated with ACTH alone [93]. These results might contradict a role for N-terminal peptides in adrenal growth, however the doses of peptides used in were supraphysiological, and a more subtle effect might have been seen if the two peptides were administered together in physiological concentrations. Furthermore, although the N-POMC(1–28) used in the study has been shown previously to be biologically active [96], the disulfide bridge arrangements in N-POMC are known to be unstable [97] and rearrangement could have occurred rendering the peptide inactive, as found in N-POMC(1–36) [89]."

ACTH, secreted by the foetal pituitary, is nonetheless proposed by many to be the primary regulator of foetal adrenocortical development. The foetal pituitary adrenal axis is postulated to become functional at 50-52 dpc, with cytoplasmic ACTH discernible, coinciding with the onset of adrenal steroidogenic enzyme expression [64]. *In vivo* ACTH stimulates steroid production by activating StAR and increasing delivery of substrate cholesterol to P450scc [98]. *In vitro* ACTH is known to promote both hypertrophy and production of DHEAS and cortisol by foetal adrenal cells [99] Furthermore, Mesiano et al have shown that foetal adrenal cells respond to ACTH by secreting growth factors that appear to stimulate

hyperplasia of the foetal zone [100] . I*n vivo,* excess ACTH is clearly involved in driving the high androgen levels seen in foetuses affected with CAH. The HPA axis clearly functions early in foetal life but the early development of the foetal adrenal in humans appears to be ACTH-independent. Normal adrenal development is observed before 8-13 wpc in anencephalic foetuses, presumed to have markedly reduced ACTH[101,102]. Thus ACTH may affect the function of the foetal adrenal at early gestation, but not its structural development. It is likely that other factors are necessary to stimulate the substantial amount of foetal adrenal steroidogenesis, particularly during the last months of gestation. Growth and rising steroid production by the foetal adrenal during pregnancy are not paralleled by increasing levels of foetal plasma ACTH (Fig. 8). A possible explanation for this paradox is that there may be gestational age-dependent alterations in responsiveness of the foetal adrenal tissue to ACTH. Alternatively there may be additional regulators of foetal adrenocortical growth and steroiodgenic function. The fact that ACTH levels do not increase significantly during the 2nd and 3rd trimesters [103,104] makes it likely that growth and differentiation of the foetal adrenal glands are influenced by placenta-derived factors during this time. This hypothesis is supported by the observation that the in the postpartum period the FZ rapidly involutes, suggesting that placental factors, which are pregnancy specific, are likely to act to maintain the FZ *in utero*.

1.5.2 Placental CRH

As described previously, hypothalamic CRH stimulates expression and processing of POMC by pituitary corticotropes and the secretion of ACTH. The human placenta, foetal membranes and decidua also express CRH that is identical to hypothalamic CRH [105]. However, unlike hypothalamic CRH, placental CRH gene expression and production can be

stimulated by glucocorticoids [106]. This positive feed-forward system is a unique feature of placental CRH, and indicates a distinct role in pregnancy.

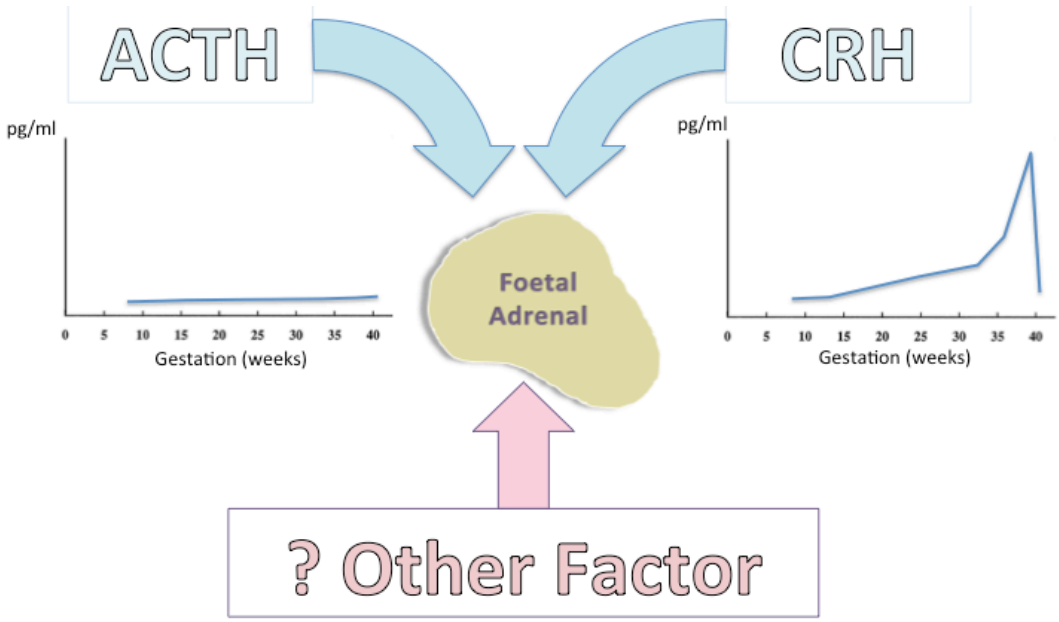
Placental CRH production is restricted to primates, and predominantly enters the maternal circulation but is also released in the foetal circulation [107]. The foetal adrenal and pituitary express CRH receptors and transcripts encoding two isoforms (CRH-R1α and –R2α) have been detected in the foetal adrenal [108–110]. CRH can directly stimulate cortisol and DHEAS production in vitro by isolated human foetal adrenocortical primary culture cells [111] by elevating the mRNA levels of steroidogenic acute regulatory protein and other steroidogenic enzymes, including *3β-HSD2, CYP21* and *CYP11B1*. In addition, CRH enhances the adrenal response to ACTH further and can stimulate expression of the receptor for ACTH in isolated DZ/TZ cells in vitro [112,113]. This suggests CRH could affect foetal adrenal responsiveness to ACTH, thereby indirectly promoting steroidogenesis, and driving the production of cortisol and DHEA/DHEAS [113].

The circulating levels of CRH increase 1000-fold as pregnancy progresses [105] and reach values of 0.5 to 1 nmol/L at term; normal values in nonpregnant women are lower than 0.01 nmol/L. Maternal plasma CRH levels are low in the first trimester, rising from mid-gestation to term. In the last 12 weeks of gestation. CRH plasma levels rise considerably, peaking during labor and then falling precipitously after delivery [114,115]. Umbilical cord blood levels and amniotic fluid levels of CRH are similarly increased in late gestation [103]. Foetal CRH levels are lower than those in maternal circulation (50 versus 1000 picomolar) but are still quite substantial compared with levels in men and nonpregnant women. CRH is the only trophic hormone releasing factor to have a specific serum binding protein. It appears that during most of pregnancy CRH-binding protein (CRH-BP) binds most of the circulating CRH in

the foetal and maternal compartment, which likely serves to tightly control the activity of placental CRH [21]. At the end of pregnancy, there is increased bioavailability of CRH, due to a fall in levels of its binding protein. This results in an exponential increase in maternal CRH levels from 35 weeks gestation to term [105,116]. Post-partum, CRH normalises to non-pregnant levels within 24 hours of delivery in keeping with the fact that the placenta is the primary source [105,116]. The step rise and peak in CRH has been proposed as an initiator of parturition, by forming a feed-forward loop that leads to increased productions of cortisol and DHEA/ DHEA-S in the HFA [116]. Indeed, women who had idiopathic preterm deliveries were seen to have higher mid-pregnancy CRH levels than those who delivered at term, and CRH has been used as a marker for risk of prematurity [105]. It could be postulated that CRH, via regulation of adrenal androgen production and hence placental oestrogen synthesis, may determine the length of pregnancy by enabling activation of parturition. Post-partum, CRH normalises to non-pregnant levels within 24 hours of delivery in keeping with the fact that the placenta is the primary source [105,116].

The rise in CRH levels with a surge late in the 3rd trimester, however are not in keeping with the rapid growth and profuse adrenocortical steroid production evident from the 1st trimester with increasing levels from the 2nd trimester to term. Although placental CRH and foetal pituitary ACTH play important roles, it is postulated that another locally produced or placenta-derived factor(s) must also be involved (Fig. 9).

Fig. 9. Proposed hormonal regulators of adrenal development



Previously proposed regulators ACTH and CRH do not fully explain the pattern of early dramatic growth and increasing steroid production demonstrated by the human foetal adrenal. This may suggest another factor with a role in regulating growth and functional development of the human foetal adrenal gland.

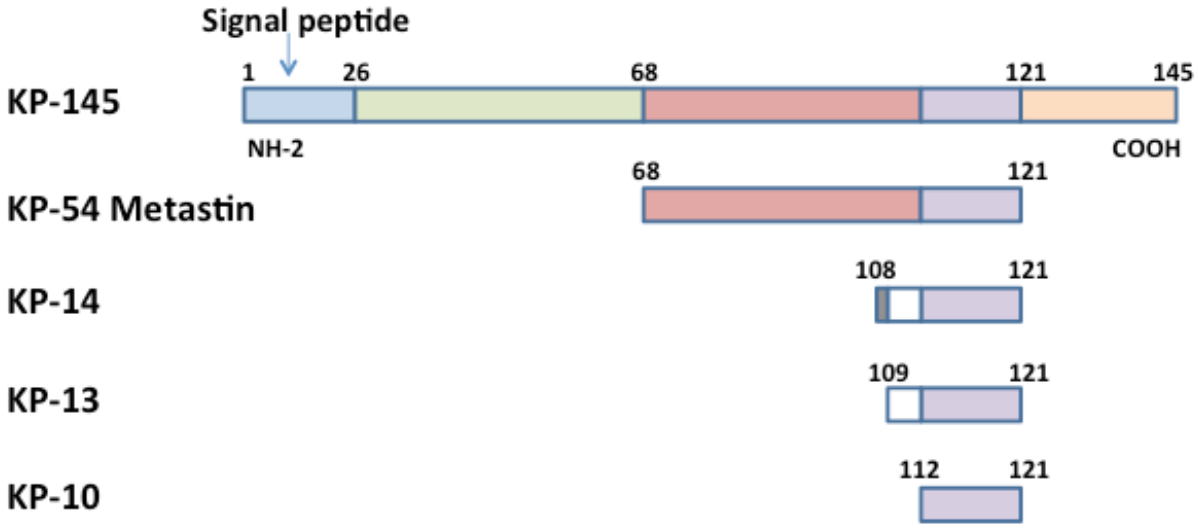
1.6 Is kisspeptin a novel regulator of adrenocortical development?

1.6.1 Kisspeptin peptides

Kisspeptins are a family of peptides encoded for by the *Kiss1* gene on chromosome 1 [117]. This family of peptides show an Arg-Phe-NH_2 motif at the carboxyl (C)-terminus, characteristic of the extensive RF-amide peptide superfamily. Post-translational cleavage of the full-length protein results in shorter biologically active fragments [118,119] (Fig. 10). The largest of these, kisspeptin (KP)-54 was initially identified in 1996 because of its ability to

suppress the metastatic potential of malignant melanoma cells, hence it was termed metastin [117,118,120]. The most abundant kisspeptin in the human circulation is kisspeptin-54, which can be further cleaved to 14, 13, and 10 amino acid peptides (Fig. 10).

Fig. 10. Kisspeptin peptides



Proteolytic processing of the full-length 145 amino acid kisspeptin protein results in shorter fragments of the carboxyl (C)-terminus region of the molecule with 54 (KP-54), 14 (KP-14), 13 (KP-13) and 10 (KP-10) amino acids.

1.6.2 Kisspeptin receptor

Kisspeptins are the endogenous ligands for a G-protein coupled receptor, discovered 4 years later than kisspeptin, and originally known as GPR54 (subsequently KISS1R in humans) [118,119,121,122]. KISS1R was initially cloned from rat brain tissue as an orphan receptor with a sequence similar to the galanin receptors, although it is unresponsive to galanin ligands [121].

All kisspeptins are able to bind KISS1R with KP-10 having maximal activity at receptor level [119]. The literature contains inconsistencies regarding the terminology applied to the kisspeptin gene and its products. However, an agreed nomenclature has been proposed [123]. In this work, the terminology suggested will be utilised, with *KISS1* and *KISS1R* denoting the kisspeptin and kisspeptin receptor gene in humans, and *Kiss1* and *Kiss1r* denoting the kisspeptin and kisspeptin receptor gene in non-primate species, respectively. The term 'kisspeptins' is also widely used for the product of both human and non-human genes [124].

1.6.3 Kisspeptin signalling

The mechanisms of action of kisspeptin are not fully elucidated. The major intracellular signalling pathway recruited by Kiss1R is the Gq pathway, which activates phospholipase C to hydrolyse phosphatidyl inositol bisphosphate to inositol triphosphate and diacyl glycerol, which mobilises intracellular calcium and activates protein kinase C respectively [122,125]. A recent study showed that the intracellular calcium release is biphasic, the first phase being rapid with the second phase being slower. The slower phase is maintained by internalization and recycling of the receptor to prevent desensitization [126].

1.6.4 Tissue distribution of kisspeptin and its receptor

Tissue distribution of Kiss1R and Kiss1 often coincides. The kisspeptin receptor has been shown to be present in the human placenta and brain [118], with quantitative reverse transcriptase-polymerase chain reaction demonstrating its abundance in the cerebellum, pituitary, cerebral cortex and brain stem [117,119,122]. Central expression of kisspeptin and its receptor have been demonstrated in two major neuronal populations within the

hypothalamus of rodents: in the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV) [127]. In humans and primates, kisspeptin mRNA is predominantly expressed within the infundibular nucleus (equivalent of the ARC in this order of mammals) [128].

In humans peripheral expression of *KISS1* and *KISS1R* genes is observed throughout the body, in the placenta, testis, ovary, small intestine, pancreas, liver, as well as the vascular system [117,118,129]. Peripheral expression of both is highest in the placenta [130].

1.6.5 Regulatory roles for kisspeptin *in utero*

Kisspeptin-expressing neurons exist in human foetuses [131]. Both *Kiss1r and Kiss1* have been identified in the hypothalamus of mice from embryonic day 13.5 onwards as well as the pre-optic area [132,133]. The presence of both the kisspeptin receptor and its ligand suggests that, this system is active from early developmental periods. Interestingly, this time point correlates with GnRH neuron migration into the preoptic area. Although conflicting evidence has been presented regarding whether kisspeptin stimulates GnRH neuron number [132], *in vitro* and *in vivo* studies have shown that kisspeptin influences GnRH neuron growth [133].

Sex differentiation is initiated during embryonic development, and although female gonadogenesis occurs autonomously, male development requires foetal testicular function with androgen synthesis [134]. The testosterone surge is thought to be critical to external genitalia masculinization. Clarkson et al showed that kisspeptin neurons are implicated in activating GnRH neurons in the male mouse during the perinatal period, and that the subsequent GnRH surge generates testosterone necessary for ensuring sexual

differentiation of the male mouse brain [135]. Kisspeptin neurons lie in close proximity to GnRH neurones suggesting a likely role in the testosterone surge [136].

1.6.6 Regulatory roles for kisspeptin in the hypothalamic-pituitary-gonadal axis

GnRH secretion is crucial for the hypothalamic–pituitary–gonadal axis, with increased pulsatility stimulating pubertal development [137]. Kisspeptin has been shown to be a potent stimulator of GnRH release *in vitro* and *in vivo*, and it has been shown to play a critical role in the onset of puberty. Administration of kisspeptin to rodents increases circulating gonadotrophins, with this effect being notably absent in *Kiss1r*–/– mice [138], suggesting that kisspeptin stimulates the hypothalamic–pituitary axis.

Two seminal papers, provided evidence that kisspeptin influences pubertal development. De Roux et al [139] looked at a series of patients with isolated hypogonadotrophic hypogonadism (IHH), a condition characterised by inadequate gonadotrophin secretion in isolation and without anosmia. The authors were able to identify a mutation affecting the *KISS1R* using a genome-mapping strategy as the responsible genetic basis for their presentation. This paper was followed by that of Seminara et al, who used linkage analysis and genetic screening in a large consanguineous family with IHH, and an unrelated patient with IHH, to identify mutations in *KISS1R* [140]. They then went on to develop a mouse model with *KISS1r*-deficient mice.

Simultaneous to the human genetic discoveries, Kiss1R- deficient mice were found to be

sexually immature with an absence of pubertal development. The animals possessed a phenotype with small gonads, and impaired spermatogenesis or no coordinated follicle maturation, presumably secondary to their low sex steroids in the setting of low circulating gonadotropins [140]. The administration of exogenous GnRH corrected the HH phenotype, which is consistent with the view that kisspeptin acts by stimulating endogenous GnRH.

These landmark findings have paved the way for a number of other studies examining mutations in the human kisspeptin receptor [141–146]. Tenenbaum-Rakover et al [146] evaluated a small series of patients presenting with IHH, all being homozygous for a single mutation leading to a leucine substitution with proline, which was found to inhibit *KISS1R* signaling. Patients were found to have LH pulses at normal frequency, but lower amplitude than expected. All patients responded to exogenous GnRH stimulation, although there was reduced pituitary response in *KISS1R*-mutated patients. The authors concluded that *KISS1R*-mutated patients have delayed pubertal maturation of the gonadotrophic axis, rather than a total absence of pubertal maturation, with pituitary and gonadal function remaining intact.

The IHH phenotype has been observed in patients with heterozygous kisspeptin receptor mutations [147], and more recently, an inactivating mutation in the kisspeptin gene in humans with absent progression of puberty has also been reported [148]. Here, a loss-of-function mutation affecting *KISS1* was responsible for the presentation of IHH in a family with four affected members. These findings suggest that both the kisspeptin receptor and a functioning kisspeptin peptide are necessary in order for kisspeptin signaling to regulate normal pubertal development. Patients with homozygous or compound heterozygous

mutations in Kiss1R fail to undergo spontaneous pubertal development and less than 30 patients to date have been described in the literature. Subsequently Teles et al. [149] identified an activating autosomal dominant mutation in the kisspeptin receptor gene in a girl with precocious puberty.

Findings from clinical research have been supported by several animal studies. Funes et al generated a mutant mouse with disruption of the *Kiss1r* receptor [150]. Phenotypic analysis showed that both male and female knockout mice had abnormalities in the development of external and internal genitalia and altered organ weight-and-body weight ratios. Lapatto et al [151] used targeted deletion to establish whether the phenotype of *Kiss1* and *Kiss1r* knockout mice would differ, revealing that although all *Kiss1* and *Kiss1r* knockout mice have abnormal sexual maturation, a subpopulation of *Kiss1* knockout females appeared to have a phenotype more similar to wild-type controls [151]. The authors postulated that although additional factors could be at play, including genetic polymorphisms, these findings indicated that mutations affecting the *Kiss1* gene could still allow a near-normal progression in rodents towards sexual maturity. It is unclear whether rodent studies can be extrapolated to humans, and studies in primates and humans would provide more translatable information.

1.6.7 Regulatory roles for kisspeptin in pregnancy

Recent evidence suggests that kisspeptin may play a role in several vital processes necessary for ensuring a successful pregnancy. Implantation has been demonstrated as requiring *Kiss1* function to ensure adequate adhesion in rodents. Calder et al described that in *Kiss1*–/– knockout mice, a failure of embryo implantation was observed due to a lack of adhesion and penetration [152]. This was found to be a function of uterine dysfunction, as evidenced by the fact that the *Kiss1*+/– embryos created could be successfully implanted in wild-type female mice.

Following embryo implantation, decidualisation occurs, characterised by stromal cell proliferation and differentiation. Zhang et al reported uterine expression of *Kiss1* and *Kiss1r* mRNA was significantly greater with decidualisation [153]. Downregulation of *Kiss1* expression using a siRNA against *Kiss1* was found to prevent the increase of *Kiss1* and *Kiss1r* demonstrated *in vitro* in a stromal cell culture model. Although the process of decidualisation is different in humans, occurring before implantation in contrast to the post-implantation process occurring in rodents [153] the findings suggested a possible mechanism underlying the relationship between kisspeptin and placental function. Evidence has also demonstrated that not only does *Kiss1* and *Kiss1r* mRNA expression increase, but that in rodents during implantation, a functional kisspeptin signalling system exists.

Having previously demonstrated phosphorylation of mitogen-activated protein kinases p38 and ERK1/2 to be a marker of activation of *Kiss1r*, Fayazi et al reported that exogenous administration of kisspeptin-54 on day 4 of pregnancy in mice led to significant activation of

kisspeptin receptors with only a weak response seen in the uteri of non-pregnant mice [154] . The authors suggested that these observations supported the notion that kisspeptin signalling is important in regulating the pregnant uterine endothelium. Although extrapolation of these findings from a small study in rodents to humans is limited, studies in man also suggest that kisspeptin is influential in pregnancy.

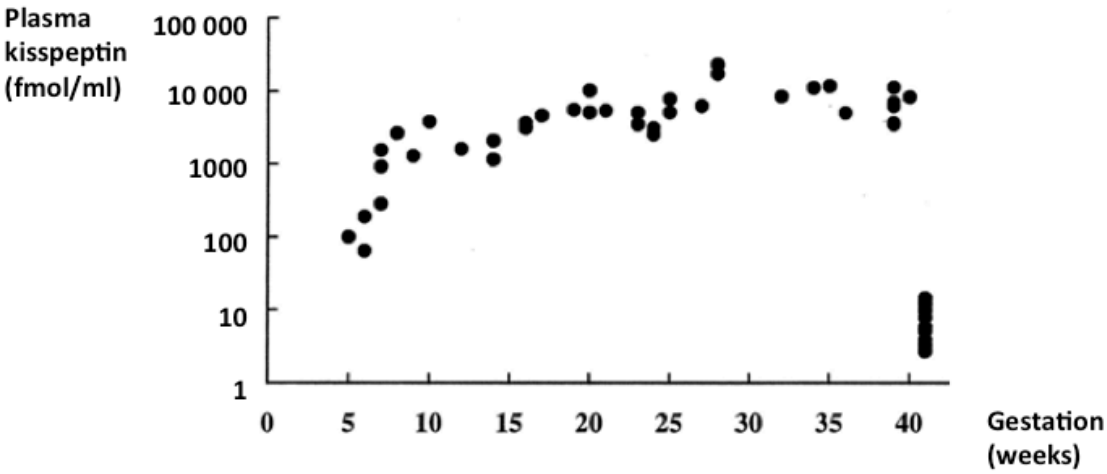
The highest levels of peripheral kisspeptin expression however, have been described in a prime location at the foeto-maternal interface comprised of the syncytiotrophoblast cells of the placenta [130]. Kiss1R has been identified in both the villous and the invasive extravillous cytotrophoblasts [130], suggesting an important role for kisspeptin in the regulation of trophoblast invasion during placentation. Using placental tissue from the first trimester and full-term delivery pregnancies, Bilban et al found placental expression of *KISS1* gene to be 29-fold higher in the first trimester human placentas compared with full-term placentas. This study interestingly showed that kisspeptin-10 appeared to block trophoblast migration, acting as an inhibitor of implantation. The authors purported that expression of kisspeptin and its receptor is greatest during the first trimester when cytotrophoblast invasion is maximal, thereby supporting the notion that kisspeptin plays a regulatory role in placentation.

Given that placentation is crucial to pregnancy outcomes, it might follow that kisspeptin expression levels alter with placental pathology. On-going studies have proceeded to investigate the potential link between kisspeptin levels and placental dysfunction such as pre-eclampsia [155], and intrauterine growth restriction [156]. Cetkovic et al. [157] found plasma

kisspeptin levels to be significantly lower in pregnant women with diabetes mellitus type 1, gestational diabetes, hypertension, pulmonary embolism, and placental dysfunction compared with healthy pregnant controls.

Circulating kisspeptin levels are low in males and non-pregnant females (<2 pmol/l) but rise dramatically by 940-fold in the first trimester, and over 7000-fold in the third trimester [158]. Levels fall rapidly by 5 days post partum, suggesting a placental source of the peptide (Fig 11) [158]

Fig. 11. Concentrations of kisspeptin during pregnancy



Levels of circulating kisspeptin rise up to 7000-fold during pregnancy, falling rapidly post delivery. (Reproduced from [158])

In support of a placental source, plasma kisspeptin levels are increased in patients with gestational trophoblastic neoplasia and fall after chemotherapy treatment [159]. Furthermore, in pregnancies complicated by pre-eclampsia, kisspeptin levels are reportedly lower, possibly reflecting placental dysfunction [160].

Not only does circulating kisspeptin appear to correlate with placental function of ongoing pregnancies, but also evidence has been provided that kisspeptin levels are lower in pregnancies that subsequently miscarry. Park et al. were the first group to suggest a link between kisspeptin and miscarriage [161]. They observed that levels of placental kisspeptin expression are lower in women with recurrent miscarriage when compared with placental tissue in electively terminated pregnancies, although no matching for gestational age was performed. Furthermore, maternal plasma kisspeptin-10 levels are lower in women with early pregnancy bleeding, suggesting a possible association with abortus imminens [162]. Jayasena et al showed that circulating kisspeptin at antenatal booking correlated with risk of miscarriage [163], with gestation-corrected kisspeptin being 60% lower in women with a singleton pregnancy compared with unaffected singleton pregnancies. The relationship of kisspeptin with miscarriage was also demonstrated in twin pregnancies, where death of one foetus was associated with a lower kisspeptin level than those without complications. Kisspeptin was also found to have a higher diagnostic performance with respect to miscarriage compared with human chorionic gonadotrophin (hCG), using receiver operator characteristic. Therefore there is compelling evidence to suggest that decreased kisspeptin may be a novel biomarker of placental dysfunction in pregnancy and may also identify asymptomatic pregnant women at greater risk of miscarriage. However, the mechanisms underlying these associations are unknown.

Pregnancy outcome data is of particular relevance given the rarity of human mutations reported in KISS1 / KISS1R. All the patients carrying homozygous or compound heterozygous mutations in this signaling system are living testimonials to their ability to establish functioning placentas during early gestation. The low frequency of human mutations in the Kiss1-Kiss1R pathway may suggest an evolutionary critical role in reproduction and species propogation that might have undergone negative selection. Brioude et al [142] described a male patient with IHH found to carry two recurrent mutations in the compound heterozygous state. Pulsatile GnRH administration restored pulsatile LH secretion and testosterone production. Later, long-term combined Gn therapy induced spermatogenesis. 3 successive pregnancies resulted in 2 miscarriages and 1 live birth. Miscarriages have also been reported in this context by Pallais et al suggesting mutations in KISS1R may affect the maintenance of pregnancy [164].

To date only one report exists in the literature of a female patient homozygous for the a missense KISS1R mutation successfully conceiving and carrying a pregnancy to term [164]. This patient underwent pulsatile GnRH therapy, with successful ovulation induction. She subsequently switched to exogenous gonadotropin therapy and had multiple conceptions, one ectopic and one miscarriage of twin pregnancy at 6 months, followed by two uncomplicated pregnancies of healthy term babies. Although much remains to be discovered regarding the intricacies of the role of kisspeptin during gestation, it is clear that it has an important function in the maintenance of pregnancy.

1.7 Thesis Rationale

HFA development and function is complex and poorly understood. Although placental CRH and foetal pituitary ACTH play important roles, other locally produced or placenta-derived factors must also be involved. Interestingly, there is 50-fold higher expression of *Kiss1R* in the HFA compared to the adult adrenal and *Kiss1R* expression has been confirmed in the DZ and TZ of the HFA [165]. Additionally, kisspeptin has been shown to stimulate aldosterone production in cultured human adrenocortical H295R cells [165]. Therefore, the HFA may be an important target for the high levels of circulating kisspeptin in pregnancy.

The objective of this project was to determine the role of kisspeptin in the developing HFA and investigate the hypothesis that kisspeptin is a novel regulator of human adrenocortical development and function, acting directly via its receptor, Kiss1R, in the foetal adrenal to regulate steroidogenesis, specifically production of DHEAS from the FZ.

The following research questions were proposed:

1. **What is the spatio-temporal expression of Kiss1R in the developing human foetal adrenal (HFA) cortex?**

2. **Is expression of Kiss1R mRNA / protein regulated by its ligand?**

3. **Can kisspeptin directly stimulate the production of DHEAS *in vitro*?**

4. **Is there a correlation between maternal serum kisspeptin concentrations and foetal adrenal growth *in vivo*?**

The first section of this thesis aims to qualitatively and quantitatively elucidate Kiss1R expression in the developing foetal adrenal. The spatio-temporal expression of *Kiss1R* in the developing HFA cortex will be examined primarily by immunohistochemical analysis of HFA sections aged 8 weeks post-conception (wpc) to term. Quantitative reverse-transcriptase PCR (qPCR) will be performed on 1^{st} and 2^{nd} trimester HFA cDNA to examine expression of *Kiss1R* with increasing gestational age.

The second section aims to investigate the effects of kisspeptin on foetal adrenal function, using two *in vitro* cell models comprising a human adrenocarcinoma cell line (H295R cells) and primary HFA cell cultures. These models will be used to interrogate the effect of kisspeptin on receptor (Kiss1R) expression and on production of DHEAS compared to other known regulators of steroidogenesis such as ACTH and CRH (measured by ELISA and LC-MS/MS).

The final section aims to ascertain whether a correlation exists between foetal adrenal growth and circulating maternal kisspeptin by studying a longitudinal cohort of pregnant women who have serial measurements of serum kisspeptin and foetal adrenal volume performed during pregnancy.

The overall aim of this work was to advance the understanding of the regulation of human adrenal development. As the foetal adrenal is a key component of the foeto-placental unit, understanding the regulation of adrenocortical development may offer insights into the regulation of the foeto-placental unit overall.

2 MATERIALS AND METHODS

2.1 Ethical approvals

2.1.1 Ethical approval for use of foetal adrenal tissues

Adrenal tissue blocks were obtained from Finland from human foetuses following therapeutic termination of pregnancy/miscarriage/stillbirth or neonatal death. Ethical approval was obtained from the ethics committee of Oulu University Hospital, Finland and a permit to study human autopsy tissues and resection material was obtained from the Finnish National Authority for Medicolegal Affairs. Use of the tissue for this project was granted ethical approval by the Brighton and Sussex NRES Committee (Rec Reference: 12/LO/1755, December 2012; Appendix 1). A MTA between the University of Kuopio and QMUL was set up to allow samples to be transferred between institutions for further studies (Appendix 2). Cryosections of frozen HFA tissue were also obtained from the HDBR (ICH, Newcastle) and a MTA between Newcastle and QMUL (Appendix 3) and UCL and QMUL (Appendix 4) was set up to allow samples to be transferred between instituitions for further studies. The HDBR was established in 1999 in line with the ethical guidelines laid out in the Polkinghorne Report (Review of the Guidance on the Research Use of Foetuses and Foetal Material, 1989). More recently, they have been licensed as a tissue bank at each site by the Human Tissue Authority. Donations of tissue are made entirely voluntarily by women undergoing termination of pregnancy. Donors are asked to give explicit written consent for the foetal material to be collected, and only after they have been counselled about the termination of their pregnancy. The HDBR can provide embryonic/foetal material from 26 post-conception days (Carnegie stage 12), up to 14 post-conception weeks. The tissue is held at the Institute for Child Health, London. It is intended for use primarily by academic researchers. The HDBR Joint Steering Committee is responsible for the overall management of the project. Tissue samples arrived at the HDBR. The head of the HDBR at ICH (Dianne

Gerelli) logged these samples and an identifying code number was assigned to each sample. The samples did not have any identifiable patient details attached to them. The gestational age of the foetal tissue will be available. Study investigators were then notified that material was available for the study.

2.1.2 Ethical approval for clinical studies

Subjects gave written informed consent, and ethical approval for these studies was obtained from the Brighton and Sussex NRES Committee (Rec Reference: 12/LO/1755, December 2012; Appendix 1). Studies were performed in accordance with the Declaration of Helsinki. Clinical studies were carried out in collaboration with teams from the Foetal Medicine Unit, Neonatal Unit and Radiology department at Barts Health NHS Trust, the Faculty of Medicine, Imperial College London, and the Steroid Lab, King's College London.

2.2 Cell culture

The human adrenocortical carcinoma cell line H295R (CRL-2128) was obtained from Ian Mason, University of Edinburgh. Primary culture of HFA cells were established as detailed later, from tissue obtained from the Human Developmental Biology Resource (HDBR), Institute of Child Health (ICH), London, and University of Newcastle. H295R cells and primary cells were grown at 37°C with 5% CO_2 in 50% (v/v) DMEM, 50% (v/v) Nutrient Mixture F12 Ham, with 2% (v/v) Ultroser G (BioSepra), 1% (v/v) ITS (containing 1mg/ml insulin, 0.55mg/ml transferrin, and 0.5µg/ml sodium selenite), and 1% (w/v) Pen/Strep [62]. All chemicals were obtained from Sigma Aldrich unless otherwise stated.

2.2.1 Trypsinisation

All reagents were warmed to 37^{o}C before use. Media was removed from the culture flask or dish and cells washed with DPBS (Dulbecco's Phosphate Buffered Saline). Trypsin-EDTA (0.5g/l trypsin, 0.2g/l Ethylenediaminetetraacetic acid; Invitrogen) was added for 30 seconds and then removed. Cells were then left for 2-5 minutes until all were detached used to detach the cells for 2-5 minutes. Fresh media was then added to the cells to neutralise the trypsin and pipetted until no cell clumps were visible. The suspension was centrifuged at 800 rpm for 1 minute to pellet the cells, which were then resuspended in fresh media and plated at the desired density.

2.2.2 Freezing down cells

Cells were grown until confluent, then trypsinsed, and transferred to a 15ml Falcon tube. They were then centrifuged at 800 rpm for 5 minutes and the media removed. The cell pellet was then resuspended in a freezing solution containing 90% (v/v) FBS and 10% (v/v) DMSO (Dimethyl Sulphoxide). 1ml of cells for each 10cm plate was then cooled at a maximum of 1^{o}C/min to -80°C in 1ml cryotubes. Tubes were then transferred to a liquid nitrogen tank for long-term storage.

2.2.3 Counting cells – Haemocytometer

After trypsinising, a 50µl sample of cells was removed and injected into the haemocytometer channel. Cells were counted in four corners of one of the grids on the slide (Fig. 12) using the Leica DMIL light microscope with 10x objective. If more than 500 cells were counted, the cell stock was diluted and another sample taken. If there were fewer than 200 cells, all four corners of both grids were counted. The following calculations were then made:

$$\frac{number\ of\ cells\ counted}{number\ of\ \text{red}\ corners\ counted} = number\ of\ cells/100nl$$

$$number\ of\ cells/100nl \times 10^4 = number\ of\ cells/ml\ (\text{y})$$

$$number\ of\ cells\ required/y = \text{volume (ml)}of\ cell\ suspension\ to\ seed$$

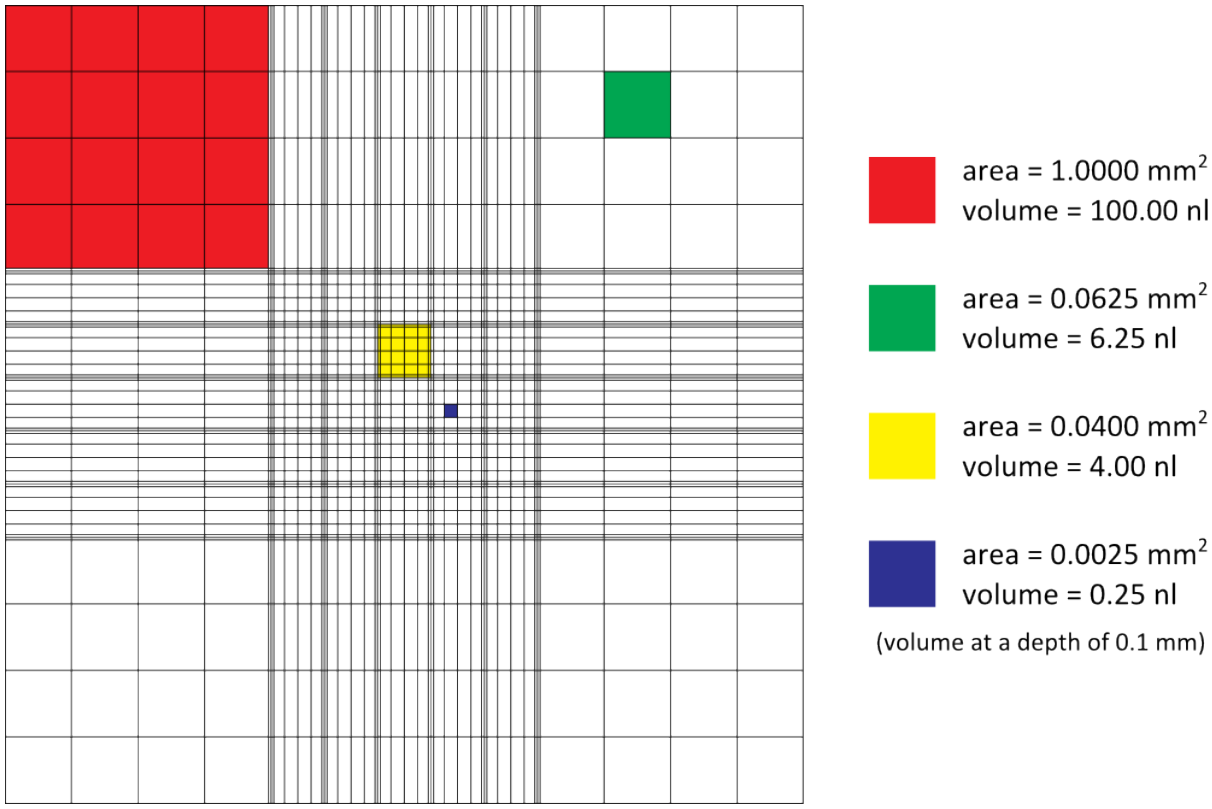


Fig. 12. Haemocytometer Grid

2.3 Primary culture of human foetal adrenal cells

2.3.1 Material

Human embryonic and foetal material was provided by the Joint MRC/Wellcome Trust [grant # 099175/Z/12/Z] Human Developmental Biology Resource (www.hdbr.org), with appropriate maternal consent and approval from the local National Health Authority Ethics Committee. The experimental protocol for this research was approved by the Brighton and Sussex REC. The samples were transported from the clinic to the HDBR resource and were staged, dissected and stored at 37°C in serum-free DMEM/F12 media with 1% (w/v) Pen/Strep. The age of the foetal samples were estimated following the guidelines of Hern [166] and the embryos were staged using the Carnegie Staging classification system [167]. A snip of tissue from each sample was taken to perform cytogenetic analysis to determine the mitotic karyotype. All samples had a normal male or female karyotype. Samples were then transported to our lab within 24 hours.

2.3.2 Collagenase digestion and establishment of primary cultures

Samples were washed in 5 ml of washing media (fresh DMEM F12, 1% (w/v) Pen/Strep) and then removed and placed in petri dish with 2-3 ml of filtered digestion media (DMEM F12, 1% (w/v) Pen/Strep, 2mg/ml collagenase). Surrounding fat tissue was removed and the gland dissociated using a sterile scalpel blade. The tissue in this digestion media was placed in a 50 ml falcon and a further 10 ml of digestion media was added. The mixture was pipetted using a 1 ml pipette and allowed to incubate in a shaking water bath at 37^0C for 1.5 hours. Every 15 minutes the mixture was pipetted using a 1 ml pipette. The mixture was then centrifuged at 800 rpm for 5 minutes and the supernatant removed. The cell pellet was then resuspended in 10 ml of fresh DMEM F12 media containing 2% (v/v) Ultroser and 1%

(w/v) Pen/Strep. The cell suspension was filtered over a cell strainer (40 µm) and the cell strainer was then washed with a further 10 ml of media. Cells were then counted as above and plated at the desired density. All flasks or plates were incubated for at least 48 hours before the first media change.

2.4 DHEAS Enzyme immunoassay

H295R or primary cells were seeded into 6-well plates and grown until 60-70% confluent. They were then incubated in serum-free media (DMEM F12, 1% (w/v) Pen/Strep) for 24 hours. Fresh serum-free media containing one of the following treatments was then added:

100 nM Kisspeptin

10 nM CRH

1 nM ACTH

10 µM Forskolin

Serum-free media (untreated control)

The doses and time period were chosen following dose-response and time course preliminary studies. *In vivo* it is estimated that the level of kisspeptin in the maternal circulation reaches a peak of ~10 nM [158]. Studies of cord blood levels suggest the kisspeptin in the foetal circulation reaches a peak of ~1 nM at term. In the maternal circulation the level of CRH reaches a peak of ~1 nM and is therefore 10-fold lower than circulating kisspeptin. The kisspeptin concentration used in experiments was supraphysiological (100x maternal circulating concentrations). This was decided on the basis of dose and time

response studies, which showed a significant increase in DHEAS after 24 hours, compared to untreated H295R cells with 10nM ($p<0.01$) and 100nM ($p<0.05$). Only 100nM kisspeptin produced a significant increase in DHEAS compared to no treatment in 8-10wpc ($p<0.05$) and 15-20 HFA ($p<0.0001$) cells. Although the concentrations of kisspeptin and CRH used in these *in vitro* experiments are higher than physiological concentrations, the ratio of kisspeptin to CRH is preserved in order to ensure that any findings can be extrapolated to an *in vivo* situation.

At the end of each experiment, the total protein content of the cells was measured, or the cells were harvested to purify RNA. The media were collected for measurement of DHEAS by enzyme linked immunosorbent assay (ELISA) from a commercial source. The DHEAS ELISA Kit (Demeditec Diagnostics, Kiel) was used. The range of the assay is between 0 – 10 µg/ml. The intra- and inter-assay coefficients of variation for all assays were <5 and 10% respectively. The assay procedure was carried out according to the supplier's instructions. Each run included a standard curve:

Standard 0 0 µg/ml

Standard 1 0.1 µg/ml

Standard 2 0.5 µg/ml

Standard 3 1.0 µg/ml

Standard 4 2.5 µg/ml

Standard 5 5.0 µg/ml

Standard 6 10.0 µg/ml

(1 µg/ml = 2.6 µmol/l)

Each microtitre well supplied was coated with a polyclonal anti-DHEAS antibody. The enzyme conjugate supplied was horseradish peroxidase. The substrate solution was tetramethylbenzidine (TMB). Stop solution contained 1 nM acidic solution. Wash solution (40x concentrated) contained 5-bromo-5-nitro-1,3-dioxane and 2-methyl-2H-isothiazol-3-one. Deionised water was added to the concentrated wash solution prior to use to obtain a 1x concentration of solution that was stable at room temperature for 2 weeks.

The desired number of Microtitre wells was secured in the holder and 25 µl of each standard, control and samples were dispensed into appropriate wells. Following this 200 µl of enzyme conjugate was dispensed into each well with thorough mixing for 10 seconds. The plate was incubated for 60 minutes at room temperature. The contents of the well were briskly shaken out and each well was rinsed 3 times with diluted wash solution. Wells were struck sharply on absorbent paper to remove residual droplets. 100 µl of substrate solution was added to each well followed by a 15-minute incubation at room temperature. The enzymatic reaction was then stopped by adding 50 µl of stop solution to each well. The absorbance of each well at 450 nm was determined within 10 minutes of adding the stop solution using the Perkin Elmer Wallac Victor2 1420 Microplate reader.

2.5 RNA Extraction

Cells grown in 6-well plates were washed with cold PBS (Phosphate Buffered Saline), then detached using a cell scraper and transferred to 1.5ml microfuge tubes. They were then centrifuged at 1200rpm for 5 minutes to form a pellet, and the PBS aspirated. The cells were lysed using 350µl of buffer RLT from the Qiagen RNeasy mini kit, containing β-

mercaptoethanol. Cells grown in 12-well plates were also washed with cold PBS, but lysed directly in the wells by the addition of 350µl buffer RLT for 20 minutes at 4°C with agitation. The lysates were then transferred to 1.5ml microfuge tubes. RNA from foetal adrenal tissue along with all other cell culture samples were extracted as described in the Qiagen RNeasy mini kit. The RNA concentration was determined by measuring the optical density of the samples at 260nm wavelength using the Nanodrop ND-1000 spectrophotometer.

2.5.1 DNase treatment

2µg RNA, 5µl 10X Turbo DNase buffer (20 mM HEPES pH 7.5, 10 mM CaCl2, 10 mM MgCl2, 1 mM DTT and 50% (v/v) glycerol; Ambion, Life Technologies), 0.5µl RNase inhibitor (40u/µl; Promega), and 1µl Deoxyribonuclease (Turbo DNase 2 units/µl; Ambion, Life Technologies) were added to a 1.5ml microfuge tube, and RNase free water (Qiagen) added to a final volume of 50µl. Samples were then briefly vortexed and centrifuged, and incubated at 37°C for 15 minutes. DNase treatment cleaves and fragments any genomic DNA contamination within the samples. The RNase inhibitor prevents RNases degrading the RNA during the treatment.

2.5.2 Phenol extraction

An amount of phenol – pH 4.7 equal to the volume of each sample was added (50µl), vortexed for 30 seconds, then centrifuged for 1 minute at 14,000rpm. The upper aqueous layer was then removed into a new 1.5ml microfuge tube, and the organic and inter- phases discarded. Phenol is an organic solvent acid used to denature and remove protein contaminants from the RNA. The pH of phenol determines the segregation of DNA and RNA between the organic phase and the aqueous phase. Acid phenol leaves RNA in the aqueous

phase. As the pH decreases, the concentration of protons increases. DNA carries a negative charge because of the phosphate groups in its sugar-phosphate backbone, which are neutralized in acid by protonation. In this case, DNA dissolves in the organic phase. RNA, however, is not neutralized in acid because, even though it also has a negative charge, it has exposed nitrogenous bases (as it is single-stranded), which form hydrogen bonds with water, thus keeping it in the aqueous phase. An acid pH also minimizes RNase activity.

2.5.3 RNA precipitation

To precipitate out RNA from the residual phenol, 3M sodium acetate buffer - pH5.3 (BDH Chemicals) was added at an amount equal to 1/10th of the volume of RNA (5µl), along with 2µl glycogen (5mg/ml; Ambion) and 125µl ethanol (2 ½ times the volume of RNA). The samples were then incubated at -20°C for 1 hour or -80°C for 20-30 minutes. After leaving for the allotted time the samples were centrifuged for 10 minutes at 4°C and 14,000 rpm to pellet the precipitate. The supernatant was then aspirated and the pellet washed with 70% ethanol to dissolve the sodium acetate. Samples were centrifuged again at 4°C and 14,000 rpm for 5 minutes, and the ethanol aspirated.

2.6 First strand cDNA synthesis

To each 2µg pellet of RNA, 12.5µl RNase free water and 0.25µl Random primers (500µg/ml; Promega) were added. They were vortexed to dissolve the RNA, and centrifuged briefly before being heated to 80°C for 10 minutes to denature the hydrogen bonds within the single stands of RNA. Samples were then cooled on ice for 1-2 minutes to allow the random primers to anneal within the secondary structure of the RNA, and given a quick centrifuge to

ensure pooling of any condensation. At this point, 1µl was removed from each sample and placed in separate PCR tubes for use as negative reverse transcription (RT) controls. A master mix containing 4µl 5X MMLV RT-buffer (Promega), 2µl DTT (Dithiothreitol 0.1M), 1µl dNTPs (deoxynucleoside triphosphates 10mM A+C+G+T; Promega), 0.5µl RNase inhibitor (40u/µl), and 1µl MMLV RT-enzyme (200u/µl; Promega) per sample was created, and 8.5µl added to each tube (total volume now 20µl). Samples were then vortexed and briefly centrifuged, then incubated at 37°C for 1 hour to allow reverse transcription to occur. After incubation 20µl of distilled water was used to dilute each sample 1 in 2. cDNA was stored at -20°C.

2.7 Polymerase chain reaction

Each 10µl reaction contained 1µl cDNA, 0.5µl dNTPs (10mM A+C+G+T), 1µl primers (10µM Forward+Reverse) (Table 1), 2µl 10X PCR buffer, 15.25µl H_2O, and 0.25µl Taq DNA polymerase (from *Thermus aquaticus*).

Standard Program:

Cycles	Temperature	Time	
1	94°C	5 minutes	strand separation = denaturation of the hydrogen bonds within the double stranded cDNA
25	94°C	30 seconds	further strand separation
	55°C	30 seconds	annealing - allows the primers to bind to a section of the single stands of DNA for which they have been specifically designed. This temperature may vary slightly depending on the T_m (melting temperature) of the primers
	72°C	30 seconds	Extension - Taq DNA polymerase binds to the primers and uses dNTPs as building blocks to enzymatically synthesise a complementary strand of DNA. The newly formed PCR product contains one strand of parental cDNA, and one newly synthesised strand. This process if repeated 25-35 times to amplify the piece of DNA between the forward and reverse primer sequences
1	72°C	7 minutes	further elongation
1	4°C	∞	Preservation

2.7.1 Gel electrophoresis

After the cDNA has been amplified, samples were combined with 5µl of loading dye (Fermentas) and run on a 1-2% (w/v) agarose gel. The loading dye increases the density of the DNA solution to contain it within the wells of the gel. The percentage gel depends on the size of the piece of cDNA that has been amplified, as higher molecular weights are separated more easily on lower percentage gels. The gel, containing 0.5µg/ml ethidium bromide, was placed in an electrophoresis chamber containing 1X TAE buffer (Tris-acetate-

EDTA buffer; National Diagnostics), and an electrical current passed through it. The PCR products were visualised using a transilluminator.

2.7.2 Oligonucleotide design

The relevant sequence of the gene of interest was established using Ensembl (http://www.ensembl.org/index.html). Primer sequences for cDNA traverse intronic regions. The following guidelines were then used for PCR primer design:

1. Oligonucleotide length of between 18 to 30 nucleotides
2. GC content should be between 40-60%
3. Primer pairs should have similar Tm (melting temperature) values, where the Tm can be estimated as follows: $T_m = 2^oC$ x (number of A and T residues) + 4^oC x (number of G and C residues).
4. Complementary sequences within a primer sequence and between pairs should be avoided.
5. Runs of 3 or more Gs or Cs should be avoided at the 3' end.
6. Complementarity of 2 or 3 bases at the 3' ends of primer pairs should be avoided to reduce primer-dimer formation.

The stock primer solution was stored at -20^oC. To avoid repeat freeze-thawing, small working aliquots of 10 **µ**M were prepared.

Table 1: Primer sequences for PCR

Primer	T_m (˚C)	Annealing Temperature (˚C)	Sequence	Product Length (bps)
Human GAPDH F	62.5	58-60	TGCACCACCAACTGCTTAG	177
Human GAPDH R	63.0		GGATGCAGGGATGATGTTC	
Reverse complement			GAACATCATCCCTGCATCC	
Human Kiss1R F	64.7	58-60	TGGTACGTGACGGTGTTCC	206
Human Kiss1R R	64.2		GCCTTCGCACTGTACAACCT	
Reverse complement			AGGTTGTACAGTGCGAAGGC	

2.7.3 Gel purification

After cutting out a band of PCR product from the agarose gel, a volume of 6M NaI (sodium iodide) three times that of the gel was added, and heated to 55°C for 5min to dissolve the agarose. 10µl of glass milk (silica in suspension in 3M NaI) was then used to absorb the nucleic acid for 5 minutes at room temperature, and a pellet of PCR product collected by centrifugation for 15 seconds at 14,000rpm. The supernatant was aspirated, and the pellet washed twice with New Wash containing 50mM NaCl (sodium chloride), 10mM TrisHCl pH7.5 (Tris Base; Fisher BioReagents, HCl; BDH chemicals), 50% (v/v) ethanol, and 7.5mM EDTA (ethylenediamenetetraacetic acid) to remove impurities. The pellet was then resuspended in 10µl distilled H_2O, heated to 55°C for 2 minutes, and centrifuged for 1

minute at 14,000rpm, to elute the PCR product from the silica. The supernatant was transferred to a new tube, and either sent for sequencing, or made up to 50µl with distilled H_2O for use in real-time qPCR. 1 µl of sample was re-run on 1% (w/v) agarose gel to confirm efficient purification. PCR products were stored at -20oC.

2.8 Sequencing

PCR products were sequenced using the ABI Prism Big Dye sequencing kit and an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA), carried out by the Genome Centre at the William Harvey Research Institute, QMUL. This is based on the Sanger dideoxy-mediated chain termination method [168], which requires a DNA template, a DNA primer, DNA polymerase, conventional dNTPs and modified nucleotides that terminate DNA strand elongation (ddNTPs). The double stranded DNA fragment is denatured into single DNA strands and a primer complementary to the known DNA sequence binds to the single stranded DNA template. DNA polymerase then binds to the primer and a new strand of DNA incorporating free nucleotides, complementary to the target DNA sequence, is synthesised. The terminating nucleotides of the ddNTPs are fluorescently labelled and modified to lack the 3'OH group required for the formation of the phosphodiester bond between two nucleotides thus terminating each DNA strand at the point of inclusion. This process is repeated many times thus generating many fragments of different lengths that all terminate in fluorescently labelled bases, corresponding to A, T, G and C. The reaction is then transferred into thin glass capillaries where application of an electrical charge moves negatively charged particles through a gel matrix and DNA fragments are then separated according to size. Finally a laser is used to excite the chain terminating fluorescent base, which is subsequently recorded as a coloured band or peak by the automated sequencer.

Analysis of sequence chromatograms was carried out using BioEdit (http://www.mbio.ncsu.edu/BioEdit/). BioEdit is a freeware biological sequence alignment editor and analysis program for Windows 95/98 NT. The program enables the user to compare the DNA sequence to a reference sequence, enabling identification of differences between 2 or more sequences by eye.

2.9 Real-time qPCR

A DNA template is replicated, as in normal PCR, but then quantified in real time by the detection of fluorescence. The amount of fluorescence recorded is proportional to the amount of DNA amplified, so DNA copy numbers, and therefore mRNA levels can be determined. The Ct value is the threshold cycle value, and the point at which amplification is first detectable above background fluorescence. A lower Ct indicates a greater amount of amplification target in the starting DNA. The Ct is measured during the exponential phase of replication, before a plateaux is reached, and is best recorded just as the fluorescent threshold has been met. A standard curve of known DNA concentrations for each gene of interest (GOI) is used to calculate the DNA copy numbers from the Ct values. The quantity of a reference RNA, such as GAPDH, is also determined to account for differences in the amount of cDNA loaded. A sample containing H_2O instead of cDNA, termed the no template control (NTC), is measured to distinguish between actual gene expression and background. The Ct of the NTC must either be undetectable, or much higher than that of the GOI.

The following criteria were used for results inclusion:

$$standard\ curve\ gradient = -3.3\ \pm 0.3$$

(This is the number of cycles expected between each standard with a serial dilution of 1 in 10).

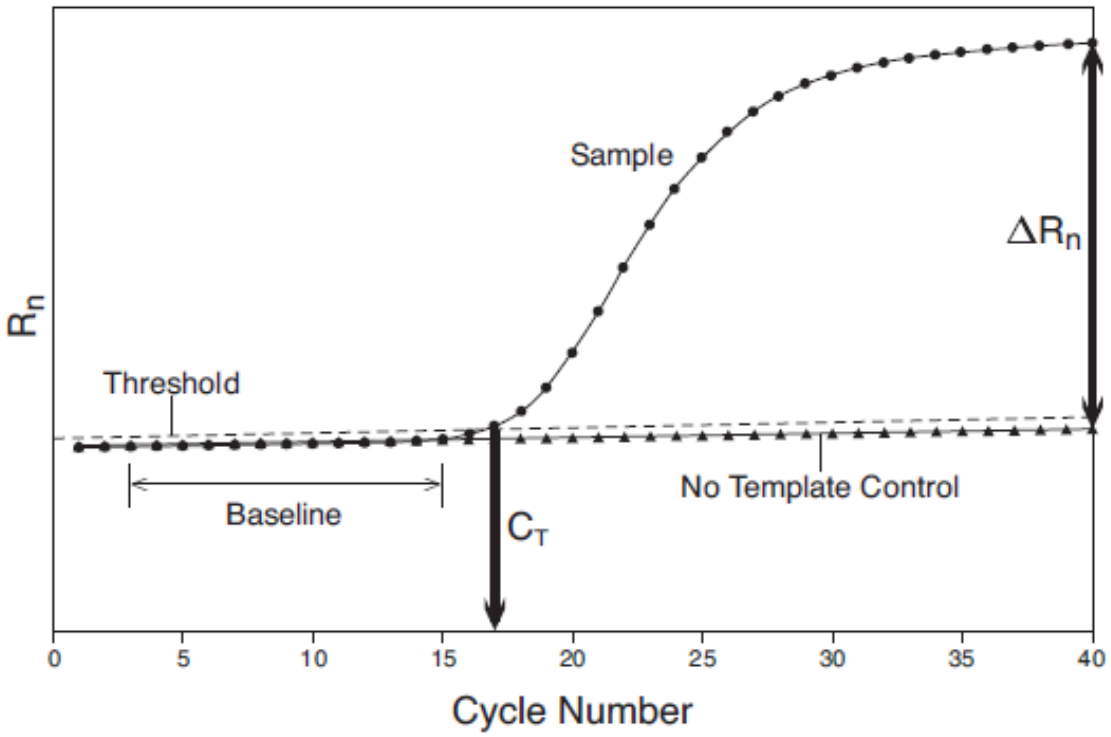
$$efficiency = 100\%\ \pm 10\%$$

$$R^2 = 1 - 0.03$$

(this indicates the relationship between the C_T and the concentration of DNA and should be linear (1)

$SYBR_{GREEN}$ is a DNA-binding dye that will fluoresce with light excitation when bound to double stranded DNA. The amount of fluorescence is therefore directly proportional to the concentration of amplified template. The emission intensity of fluorescence during the PCR reaction is plotted against the cycle number to produce a sigmoidal amplification plot (Fig. 13a.).

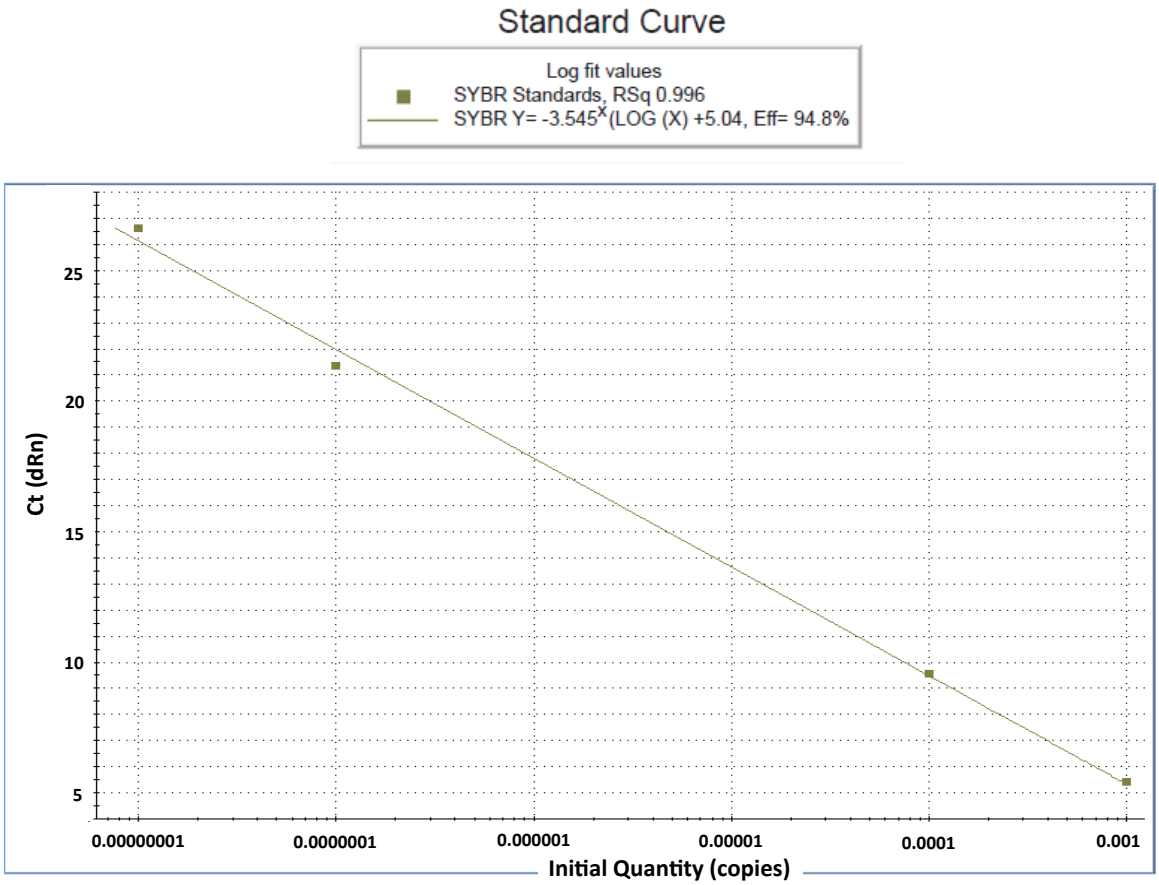
Fig. 13a. Amplification plot (Applied Biosystems technical manual 4309155)



Reactions were run in duplicate or triplicate in a 96-well plate, including a NTC. Plates were centrifuged at 1500 rpm to remove bubbles and ensure the reaction mixture was at the bottom of the wells. Standard curves were created using serial dilutions of a known concentration of DNA for the gene of interest and of the internal control (GAPDH). In the initial cycles of PCR, there is little change in fluorescence signal and an increase in fluorescence above baseline indicates the detection of an accumulated target. Amplification plots are the plots of fluorescence signal versus cycle number. A fixed fluorescence threshold is set in the region associated with exponential growth of the PCR product. The threshold cycle (Ct) is defined as the fractional cycle number at which fluorescence passes the fixed threshold. The higher the starting copy number of the nucleic target the sooner a significant increase in fluorescence is observed. The no template control (NTC) is a sample that does not contain template and is used to verify amplification quality. The Ct of the NTC should be more than 5 cycles away from that of the target. Dissociation curve analysis is

used to determine the melting temperature (Tm) of the PCR product. The Tm is characteristic of the GC content, length and sequence of a DNA product and can be used to establish that the correct DNA product has been amplified. The standards of the gene of interest have the same Tm, with the NTC melting at a lower temperature. The standard curve is generated from the Cts of the standards (Fig. 13b). Adequate PCR efficiency has to be achieved with a minimum of 3 replicates and a minimum of 5 logs of standard template concentration. A slope of -3.3 +/- 10% reflects an efficiency of 100% +/- 10%. A PCR reaction of lower efficiency will have lower sensitivity. If the standard curve is of appropriate efficiency it can then be used to extrapolate the quantity of the target.

Fig. 13b. Representative image of a standard curve generated from the Ct of the standards, from which the quantity of a target can then be extrapolated.



This method of generating a fluorescent signal is cheap and easy to use, as the same dye can be used for all genes. However, this does mean it lacks in specificity, so the quantity of only one gene per well can be measured. Amplification in the NTC is also often seen, as it cannot distinguish between real template and artefact bands, such as those created by primer-dimers.

Each 10 µl reaction contained 2 µl cDNA template, 5µl 2X $SYBR_{GREEN}$ I master mix (KapaBiosystems), 0.2 µl low ROX (KapaBiosystems), 0.5 µl primers (10 µM Forward+Reverse), and 2.3 µl sterile H_2O. The 2X $SYBR_{GREEN}$ I master mix contains the $SYBR_{GREEN}$ I fluorescent dye, $MgCl_2$, dNTPs, stabilisers, and DNA polymerase. ROX is used as a reference dye as it does not intercalate with DNA and increases its fluorescence during the PCR reaction. This is used as an internal reference to correct for inter-well fluorescence fluctuations and for evaporation during cycling. See Table 1 for list of primers.

Using the MX4000 real time thermocycler (Stratagene), a quantitative method was selected.

Quantitative fast cycle program:

Cycles	Temperature	Time	
1	95°C	3 minutes	strand separation
35	95°C	3 seconds	
	60°C	30 seconds	annealing and extension
	72°C	1 second	
1	95°C	1 minute	
1	60°C	30 seconds	dissociation melt
1	95°C	30 seconds	

The dissociation melt separates double stranded DNA back to its single stranded form. As different genes have different melting temperatures, the quality of the amplification product can be assessed. All wells amplified with the same primers should contain the same cDNA, with only one peak detected at a temperature corresponding to the amplification product's T_m. Primer-dimers, or amplification of more than one DNA template may result in a second smaller peak.

The quantitative protocol displays all DNA copy numbers, but comparisons must be done separately after dividing the GOI values by the reference gene values.

2.10 Protein analysis

2.10.1 Protein extraction for Western blotting

To prepare lysates, media was removed by pipetting and cells were washed in cold PBS. For a 6 well plate cells were lysed in 200 μl RIPA buffer (Sigma-Aldrich) containing protease inhibitor (complete, Mini, EDTA-free Protease Inhibitor Cocktail tablets, Roche). They were then scraped off the plate and transferred to 1.5 ml eppendorf tubes. Tubes were left on ice for 30 minutes, then centrifuged at 13 000 rpm for 12 minutes at 4^{o}C to pellet cell debris. Supernatant was collected and added to an equal volume of 2x Laemmli buffer (Sigma-Aldrich), containing SDS and β-ME, then boiled for 10 minutes to denature proteins prior to gel loading.

2.10.2 Bradford protein assay

The Bradford protein assay (Bradford, 1979) was used to determine protein concentration of cell lysates. The assay is based on the maximum absorbance shift from 465nm to 595nm of Coomassie Brilliant Blue G-250 (5x) (Biorad) protein assay dye when bound to protein. Serial dilutions of bovine serum albumin (BSA) (Sigma-Aldrich) ranging from 0 – 1mg/ml in concentration were used as protein standards. The protein assay dye was diluted 1 in 5 with PBS prior to the assay. 10 μl of standard or test sample were added in triplicate to each well of a 96 well plate, together with 200 μl of protein assay dye. The samples were left to incubate at room temperature for 5 minutes. Absorbance of each well at 595 nm (OD595)

was determined using the Perkin Elmer Wallac Victor2 1420 Microplate reader. A standard curve was produced by plotting the OD595 against concentration of the BSA standard, and the protein content of each sample was determined by extrapolating from the linear equation as previously described (Bradford 1979).

2.10.3 Western blotting

A pre-cast gel (SDS PAGE precast NuPage BisTris gels (w/v) 4-12%, 10% or 12%) (Invitrogen) was briefly rinsed with distilled water and inserted into the gel tank. The tank was filled with 1x MOPS running buffer (50x 3-(N-morpholino) propanesulphonic acid (50nM MOPS, 50nM Tris Base, 0.1% (w/v) SDS, 1mM EDTA pH 7.7)) to cover the gel and the comb removed the protein ladder (Kaleidoscope prestained Protein Marker (Biorad) was loaded into the first well of the gel (10 μl for 10 well gel, 8 μl for 15 well gel). Samples prepared in 2x Laemmli buffer were centrifuged for 1 minute at 13 000 rpm and loaded into adjacent wells, typically between 20-30 μl, depending on the protein content of the lysate. The gel was run for 30 minutes at 70V and an hour at 160V.

2.10.4 Semi-dry transfer of proteins from gel to membrane

A square segment of 0.1 μm pore nitrocellulose membrane was cut to the dimensions of the gel using extra thick filter paper (Biorad) as a template. The membrane was soaked in transfer buffer (0.9% glycine (w/v), 0.242% Tris (w/v), 20% methanol (v/v)) for 10 minutes prior to the transfer procedure. A sandwich of filter paper-membrane-gel-filter paper was assembled in the transfer cell. First, filter paper was briefly soaked in transfer buffer and placed on the transfer cell, pressed with a roller to remove excess buffer. Next the

membrane was placed on top of the filter paper and then the gel was soaked and placed on top of the membrane. Bubbles were removed by gently pressing the gel from the centre to the corners. Finally the second filter paper was soaked and placed on top of the gel. The sandwich was pressed again with the roller to remove excess buffer. The transfer cell (Trans-Blot SD semi-dry transfer cell) (Biorad) was set to 15V for 40 minutes for one blot or 50 minutes for two blots. After transfer the gel was discarded and the membrane subjected to Ponceau-S staining to determine the efficiency of the transfer.

2.10.5 Ponceau-S staining

Ponceau-S (0.2% (w/v) Ponceau-S and 1% (w/v) acetic acid) (Sigma-Aldrich) is a dye that reversibly stains protein bands without any adverse effects on the proteins. Staining membranes with Ponceau-S reveals any regions where the transfer has failed (seen as bubbles) as well as the amount of protein loaded per lane and the efficiency of the transfer. Membranes were placed in small containers, covered with Ponceau-S and left on a shaker for about two minutes. Ponceau-S was removed and the membrane washed with PBS-tween (1x PBS +0.1% (v/v) Tween20) (Sigma-Aldrich). After one wash, protein bands became visible, and Ponceau-S staining was then removed with further washes in PBS-tween.

2.10.6 Immunoblotting

A blocking buffer was prepared by dissolving 5% (w/v) non-fat dried powdered milk (Asda) in PBS-tween (0.1% (w/v)), and mixing by inversion. Nitrocellulose membranes were transferred to 50 ml falcon tubes with 10 ml blocking buffer for one hour to prevent non-specific binding of the antibody. Tubes were rotated on a roller. After blocking, membranes

were incubated in milk containing relevant dilutions of primary antibodies (Table 2) overnight at 4^{o}C. The following day, membranes were subjected to 3x 10 minute washes in PBS-tween, incubated in milk containing fluorophore-conjugated secondary antibodies (Table 2) (goat anti-rabbit 800 1:5000, goat anti-mouse 680 1:20 000) (Odyssey) for one hour and wash steps were repeated. Bands were detected using an Odyssey scanner at wavelengths 800 and 680.

Table 2. Antibodies used for western blotting

Ab	Antigen/Fluorophore	Species	Company	Dilution
1°	β-actin	Mouse monoclonal	Sigma-Aldrich	1 in 10 000
1°	Kiss1R	Rabbit polyclonal	Alomone AKR-001	1 in 500
2°	IRDye®680LT	Goat anti-mouse	Li-cor	1 in 20 000
2°	IRDye®800CW	Goat anti-rabbit	Li-cor	1 in 5000

2.11 Cytology

2.11.1 Fixation

Cells grown on cover slips were washed twice with ice cold PBS, and fixed for 15 minutes in 4% (w/v) PFA (paraformaldehyde; BDH Chemicals) on ice.

PFA holds the cells architecture in place by creating chemical cross-links that join proteins via their amino side chains.

2.11.2 Immunofluorescence

After fixation, cells were blocked with 10% (v/v) normal donkey or goat serum in PBS-triton-Na azide containing 0.1% (w/v) triton X-100 and 0.2% (w/v) Sodium azide (Fluka BioChemika), then incubated overnight at room temperature with primary antibodies (Table 3), also in PBS-triton-Na azide. Residual unbound primary antibodies were removed by washing the cells three times in PBS with 0.1% (w/v) triton X-100 (PBS-triton) for 10 minutes each. Incubation with secondary antibodies (Table 3) in PBS-triton was carried out for 3 hours at room temperature, before repeating the washes in PBS-triton to remove any unbound secondary antibodies. One drop of DAPI (4',6-diamidino-2-phenylindole) was then added to each cover slip for less than 1 minute before being washed away. Cover slips were transferred onto microscope slides, and secured with fluorescent mounting media (Dako). Slides were visualised, and images taken using the Zeiss LSM510 laser scanning confocal microscope, and the ZEN 2011 Light edition software.

Table 3. Antibodies used for immunofluorescence

Ab	Antigen/Fluorophore	Species	Company	Dilution
1°	SF1	Mouse monoclonal	Invitrogen 434200	1 in 200
2°	AF488-Green	Goat anti-mouse	Invitrogen A11029	1 in 1000
1°	Kiss1R	Rabbit polyclonal	Alomone AKR-001	1 in 100
2°	CY3-Red	Donkey anti-rabbit	Jackson Immunoresearch 711-165-152	1 in 1000
1°	SULT2A1	Rabbit polyclonal	Abcam 38416	1 in 200
2°	CY3-Red	Donkey anti-rabbit	Jackson Immunoresearch 711-165-152	1 in 1000
1°	CD56-conjugated AF488-Green	Mouse monoclonal	Invitrogen MHCD5620	1 in 1000

2.12 Histology

2.12.1 Sectioning and Mounting

10µm thick sections were cut from adrenals embedded in paraffin using the Leitz 1512 microtome, and mounted on TESPA treated slides (3-triethoxysilylpropylamine; VWR) by the floating-out technique. Briefly, a small amount of water was placed on the slides, and ribbons of serial sections laid flat on the water. They were heated to approximately 38°C, and the water removed by pipetting from a corner or edge, leaving the sections to bond with the adhesive coating (TESPA). The slides were then dried overnight at 37°C to increase the adhesion, and ensure the sections would not dissociate during further treatments.

2.12.2 Deparaffinisation

As paraffin is insoluble in water, most staining techniques require it to be removed. To deparaffinise, sections were washed three times in xylene, twice in 100% ethanol, once in 90% (v/v) ethanol, once in 70% (v/v) ethanol, once in 50% (v/v) ethanol, and then rehydrated by washing twice in H_2O. Each wash lasted 10 minutes.

2.12.3 Haematoxylin & Eosin (H&E) staining

Slides were washed for 2 minutes in haematoxylin (Lamb Laboratories), and 2 minutes under running H_2O. They were then immersed in acidic alcohol 10 times (75% (v/v) ethanol, 0.04% (v/v) HCl), followed by another 2 minutes under running H_2O. After immersing the slides 10 times in ammonia solution (0.084% (v/v) ammonium hydroxide), they were subjected to 5 minutes under running H_2O, 10 immersions in 80% (v/v) ethanol, placed for 15 seconds in Eosin (Lamb Laboratories), followed by two lots of 10 immersions in 95% (v/v) ethanol, two lots of 10 immersions in 100% ethanol, and two 10 minute washes in xylene. Excess xylene was wiped from the back and edges of the slides, and they were covered with glass slips, using DPX mounting media (Lamb Laboratories) to preserve the staining. They were visualised and imaged using the NanoZoomer-2.0 HT Digital Slide Scanner (Hamamatsu) and Panoramic Viewer (3DHistech) software, and the Leica DMR Light microscope, Lieca DC200 digital camera, and Leica DCViewer software.

2.12.4 Immunofluorescence

Tissue from early gestation (8 – 13 wpc) was studied in collaboration with Achermann's group at ICH using material collected from the HDBR. Paraffin embedded wax sections were studied both in our lab and at ICH.

Paraffin sections were deparaffinised with three washes in xylene. Ethanol washes were continued using 100%, 90%, 70% and 50% (v/v) solutions, followed by two washes with H_2O to rehydrate. The slides were then boiled for 15 minutes in 10mM sodium citrate buffer - pH6 (BDH Chemicals) to break cross-links formed between proteins during paraffin embedding, and unmask antigens and epitopes. Cryosections were left at room temperature for 10 minutes. After a 5-minute wash in PBS-triton, the slides were blocked for 30 minutes with 10% (v/v) normal goat serum. The primary antibody (or control solution) (Table 3) was applied overnight at room temperature, then any unbound removed by washing three times in PBS-triton. Negative controls were included in experiments to ensure staining was specific. Controls comprised omission of the primary antibody, or pre-incubation of the primary antibody with its peptide antigen. Pre-incubation required addition of the blocking peptide (at a final concentration of 1μg/ml) to a solution containing the primary antibody, which was then agitated at room temperature for 30 minutes. This was followed by 2 hours incubation with the secondary antibody (Table 3). One drop of 4',6'-diamino-2-phenylindole (DAPI, Vector Laboratories, Peterborough, UK)) was then added to each cover slip for less than 1 minute before being washed away. Cover slips were secured with fluorescent mounting media (Dako). Slides were visualised, and images taken using the Zeiss LSM510 laser scanning confocal microscope, and the ZEN 2011 Light edition software.

2.13 Non-radioactive *in situ* hybridisation

2.13.1 RNA probe design and labelling

cDNA from foetal adrenal tissue was prepared as described above following RNA extraction using RNeasy Mini kit (Qiagen), using random-primed total RNA using Moloney Murine Leukaemia Virus-Reverse Transcriptase (MMLV-RT), Promega). Kiss1R cDNA fragments were PCR amplified using the following primers (Table 4)

Table 4. Primers used for RNA probe design

Primer	T_m (˚C)	Annealing Temperature (˚C)	Sequence	Product Length (bps)
Human Kiss1R F	61.99	58-60	CCCCTTCCTGAGTTCCACAG	932
Human Kiss1R R	61.98		GGCGAAGAGCAGGACCAC	
Reverse complement			GTGGTCCTGCTCTTCGCC	

2.13.2 RNA probe labelling

Amplified PCR products were cloned into the dual promoter vector pGEM-T easy (Promega, Southampton, UK) and linearized with the appropriate restriction enzymes. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were synthesized by *in vitro* transcription in the presence of DIG-labeling mix ((Roche Diagnostics Ltd, West Sussex, UK) using ~1 µg of

linearized template and T7 or SP6 RNA polymerase (New England Biolabs). The concentration and integrity of the RNA probe was analyzed by gel electrophoresis and spectrophotometrically; the transcription reaction was diluted with diethylpyrocarbonate (DEPC)-treated H_2O to a concentration of 100ng/µl transcript, aliquoted, and stored at -80°C. The probe was used at a concentration of 400ng/ml of hybridization buffer. The probe was designed to include regions of low nucleotide identity with other related family members or other sequences located in the NCBI nucleotide database.

The *in situ* protocol was performed as in Guasti *et al* [42]. Cryosections of HFA tissue were fixed in 4% (w/v) PFA. All were then permeabilised with Proteinase K (Sigma Aldrich, Poole, UK, 5ug/ml in 100mM Tris HCL pH 7.5 and 50mM EDTA, pH 8.0) for 10 minutes at 37°C. After re-fixing in 4% (w/v) PFA and 3 washes, sections were hybridized overnight at 58°C with either sense or antisense riboprobes in incubation buffer containing 50% (v/v) formamide, 0.3M NaCl, 10mM Tris-HCL pH7.5, 1mM EDTA, 5% (w/v) dextran sulphate, 1 x Denhardt's solution, 0.5mg/ml denatured salmon sperm DNA and 0.02% (w/v) SDS. Slides were then washed through a series of saline-sodium citrate (SSC, National Diagnostic) buffers (2x SSC, three times each for 30 min, and once each in 1x SSC, 0.2x SSC and 0.05x SSC for 15 min, respectively) at 65°C. They were then washed in STE buffer (0.5M NaCl, 10mM Tris pH 7.5, 5mM EDTA) for 10 min at RT followed by treatment with RNase A (QIAGEN, 25ml of 20mg/ml stock in 50 ml of STE buffer) for 30 minutes at 37°C. After washing the slides twice with Maleic Acid Buffer (MAB) (0.1M maleic acid, 0.15 NaCl, pH 7.5, 0.1% (w/v) Tween) they were incubated with anti-DIG alkaline phosphatase-Fab fragments (Roche Diagnostics Ltd, West Sussex, UK) diluted 1:2000 with 0.5% (w/v) Blocking Reagent (Roche Diagnostics Ltd, West Sussex, UK) in MAB/0.1% (w/v) Tween 20, overnight at 4°C.

After two washes with T-PBS the slides were equilibrated in alkaline buffer (100mM Tris pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 1% (w/v) Tween-20) for 10 min before being incubated at room temperature with NBT/BCIP (Roche Diagnostics Ltd, West Sussex, UK) in alkaline buffer supplemented with levamisole (Vector Laboratories, Peterborough, UK; 1drop/5ml). Sections were checked every hour until there was adequate staining. Sections were finally washed in T-PBS and mounted with a glycerol-based mounting medium (glycerol/PBS, 3:1 v/v).

2.13.3 Image acquisition

Images were acquired using a Leica DM5500B microscope (Leica, Nussloch, Germany), equipped with a DCF295 camera (Leica) and DCViewer software (Leica), and then processed with Abode Photoshop CS6 and Adobe Illustrator CS6.

2.14 Liquid chromatography-tandem mass spectrometry

DHEAS was measured in the cell media by enzyme linked immunosorbent assay (ELISA, as described above) (Demeditec Diagnostics, Kiel) and checked for some experiments by analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS). This work was performed by collaborators at the Steroid Lab, King's College Hospital (Dr Lea Ghataore, Dr Norman Taylor). LC-MS/MS is the gold standard method for quantifying steroid production. Mass spectrometry results were unavailable for all the time points of interest.

2.14.1 Sample preparation

500µl of precipitation reagent (a cocktail of internal standards in acetonitrile, including 16,16 d_2 DHA sulphate) was added to the samples, calibrators and internal quality controls (500µl). The quality controls were created in-house from a standard solution of DHA sulphate diluted in charcoal-stripped serum. Samples were vortexed (30 seconds) and centrifuged (13 000rpm) for 5 minutes. The supernatant was transferred into glass tubes. Bicarbonate solution (200µl, 8% aq, v/v) and 1ml of ethyl acetate was added and the tubes were vortexed (30 seconds) and centrifuged (13 000 rpm) for 5 minutes. The organic layer was transferred into a glass tube and evaporated to dryness under nitrogen gas and reconstituted in 125µl of freshly prepared reconstitution solution (Mobile phase A: Mobile phase B, 65:35 (v/v)). 100µl was injected onto the liquid chromatography (LC) system. Using the TSQ Vantage (ThermoFisher) in MS/MS positive APCI mode, *m/z* transitions 271.1 to 105 & 91 were monitored. DHEA was also quantified in the same runs and remained near or below detection limits, indicating that there was no significant desulphation during sample processing.

2.14.2 Liquid chromatography conditions

Eluents:	Mobile Phase A:	Water with 0.1 % (v/v) formic acid
	Mobile Phase B:	Methanol with 0.1 % (v/v) formic acid
Flow rate:		0.4ml/min
Column:		Accucore RP-MS Column (100 x 2.1mm. 2.6 µm)
Column temperature:		40 ºC (maintained by Hot Pocket. ThermoScientific
Detector:	MS/MS (positive APCI, 2 *m/z* transitions per analyte are monitored)	
	Model:TSQ Vantage (ThermoFisher)	

2.14.3 Mass spectrometry parametes

Vaporizer temperature	**500 °C**
Capillary temperature	400 °C
Discharge current (mA)	5.0
Sheath gas	20
Auxillary gas	5
Collision gas pressure (mTorr)	1.5
Q1 (FWHM):	0.40
Q3 (FWHM):	0.70
Scan time	0.05 seconds

2.14.4 DHEAS assay validation data

Precision and accuracy

Concentration nmol/L (n=6)	**102**	**204**	**1697**	**8148**
Intra-assay				
Mean	99.8	187.3	1840	8080
Accuracy %	97.9	92	108	99
CV%	5.3	3.0	3.8	1.3
Inter-assay				
Mean	102.3	201.7	1789.7	7700.8
Accuracy %	100.3	98.9	105.5	94.5
CV%	8.1	3.7	5.6	4.7

Lower limit of quantification is 35 nmol/L

No carry over or carry under was detected

No Ion suppression/enhancement of DHEAS-d2 signal was noted

Reagent stability

Concentration nmol/L (n=3)	204	1697	5729
Post extraction 1 week @4C			
Mean	201.4	1646	5720
Accuracy %	96.7	97.0	99.8
Post extraction 1 week @RT			
Mean	202.4	1570	5539.3
Accuracy %	99.2	92.5	96.7
3 freeze-thaw cycles			
Mean	203.2	1633	5581
Accuracy %	99.6	96.2	97.4

2.15 Clinical study

2.15.1 Clinical study design and recruitment

A prospective observational study of patients with singleton, uncomplicated pregnancies was undertaken in collaboration with the Foetal Medicine Unit at the Royal London Hospital (Dr Rebecca Allen, Dr Shemoon Marleen, Mr Joseph Aquilina). Women attending their routine antenatal ultrasound scan (USS) at ~12 weeks gestation at the Royal London Hospital, London between February 2013 and April 2014 were recruited.

I designed this clinical study with the intention that a case group of pregnant women who go on to develop pre-eclampsia (PE), and a control group of pregnant women with uncomplicated normal pregnancies would be recruited in the first trimester. Pre-eclampsia is a condition associated with placental dysfunction, and therefore presumed lower levels of placental hormones such as kisspeptin. As discussed previously the association of PE and low maternal kisspeptin levels has been reported in the literature [160]. Biomarkers previously described to be associated with the onset of PE, particularly early onset disease, include placental growth factor (PlGF) and β-hCG. PlGF is a polypeptide growth factor and a member of the vascular endothelial growth factor (VEGF) family, which regulates the development of placental villi during early gestation. This regulatory effect is essential for establishing placental blood circulation to ensure a normal pregnancy outcome. In normal pregnancy PlGF steadily increases during the first two trimesters and peaks at 29-32 weeks and then declines. Previous studies have reported PE in association with low placental growth factor (PlGF) [169,170]. In PE, PlGF concentrations start to decrease 9-11 weeks before the onset of hypertension and proteinuria [170]. hCG is produced by the syncitiotrophoblast of the placenta and consists of alpha and beta subunits. It has been postulated that low β-hCG between 10-14 weeks may be a consequence of impaired placentation and a smaller placental mass and that subsequently high 2nd trimester levels may develop as a result of hypoperfusion–related stimulation [171,172]. Several previous studies have reported an association between increased 2nd trimester levels of β-hCG and adverse pregnancy outcomes including PE. Other studies report that decreased levels of β-hCG in the 1st trimester were predictive of PE [171]. Abnormal 1st trimester levels of β-hCG have also been reported as not significantly associated with any adverse outcomes, including PE [156,173]. PE affects around 5% of pregnancies and is a major cause of maternal and perinatal morbidity and mortality [174]. It is characterised by pregnancy induced hypertension, blood pressure ≥140/90mmHg, and

protein in the urine (0.3g/24h) (+/- pathological oedema) after 20 weeks gestation. PE is associated with poor placentation and incomplete remodelling of the utero-placental spiral arteries. In pregnancies complicated by PE, placental abruption and birth of SGA neonates, there is histological evidence of impaired trophoblastic invasion of the spiral arteries. PE is typically diagnosed in the late 2nd or early 3rd trimester. More recently abnormal uterine artery Doppler in the 1st trimester was shown to identify a high-risk group of women who could be targeted for closer, more intensive antenatal surveillance and clinical management [175]. The specificity for predicting early onset pre-eclampsia using this screening method was reported to be high. First trimester uterine artery Doppler is therefore a useful tool to stratify women by risk status and target them for appropriate clinical management and identify higher risk individuals for research purposes. A research study carried out by collaborators in Foetal Medicine Unit at the Royal London Hospital in 2011 (Mr Joseph Aquilina, unpublished) revealed that 20% of women scanned at 12 weeks had abnormal uterine artery Doppler measurements. Out of this group, 35% of those with abnormal 12-week Doppler measurements went on to develop PE. The remaining 65% of women screened have uncomplicated normal pregnancies. The design of this study incorporated this information, anticipating that the finding of abnormal Doppler measurements at 12 weeks would provide both a cohort of women with normal pregnancies and a cohort that go on to develop PE. The advantage of using this starting point for selection and enrolment was that both cohorts would receive exactly the same follow up during the course of the study.

Eligible candidates were identified by Obstetric collaborators (Dr Rebecca Allen, Dr Shemoon Marleen) at the 12-week antenatal booking visit as those with a singleton pregnancy and a uterine artery pulsatility index >1.96 on Doppler ultrasound. Participant information sheets were produced (Appendix 5) and written informed consent (Appendix 6,

consent forms) was obtained for all participants and translators were provided where necessary. The GP of participants were notified of inclusion in the study with consent (Appendix 7, GP letter).

2.15.2 Patient selection

Inclusion criteria:

1. Singleton pregnancy
2. PI > 1.96 on ultrasound at 12 weeks gestation

Exclusion criteria:

1. Multiple pregnancy
2. Coexistent maternal medical conditions (pre-existing hypertension, diabetes, thyroid, adrenal or renal disease)
3. Congenital foetal abnormalities or chromosomal anomalies
4. Maternal infection (including HIV)
5. Maternal alcohol abuse
6. Heavy smoking (>10 cigarettes daily prior to pregnancy; >5 cigarettes daily during pregnancy)
7. Maternal exposure to psychotropic medications.

2.15.3 Source of patients

Subjects were recruited from the following cohort of patients: Pregnant women who present to the antenatal clinic at the Royal London Hospital and had already consented to have their routine 12 week scan performed by a senior obstetric doctor (collaborators from the Foetal Medicine Unit, Royal London Hospital) as part of a study examining Doppler measurements. Gestational age was established during the USS evaluation.

2.15.4 Informed consent procedures

Written information about this study (Appendix 5) was provided at 12-week scans by foetal medicine collaborators (Dr Rebecca Allen, Dr Shemoon Marleen) once the patient has given verbal consent to receive information about the study. If translation was required, the information leaflet was translated using the Barts Health NHS Trust translation service. Information about the study was given to the patient at least 6 weeks before written consent was obtained in order to ensure adequate time for potential participants to understand the commitment to the study and to address any questions they had. Written informed consent (Appendix 6) from those patients, who agree to participate in the study, was obtained by a member of the research team (myself, Dr Muriel Meso, Miss Shezhan Elahi) at the time of the anomaly scan at 18-22 weeks. This followed adequate explanation of the aims, methods, anticipated benefits and potential hazards of the study. It was also made clear to the potential participant that they were free to refuse any involvement within the study or alternatively withdraw their consent at any point during the study and for any reason. The participants' GP was informed with consent of a participant's involvement in the study (Appendix 7). The Consultant Clinician and Obstetric team responsible for the patient's antenatal care were also made aware of participation in the study by highlighting

this in the patient's medical records along with signed copies of the consent form and study information leaflet.

2.15.5 Premature withdrawal / loss to follow up

Any patients lost to follow up or who withdrew consent and no longer wished to participate in the study at any stage were included in the data analysis. For patients who, during the course of the study, no longer fit the initial inclusion criteria, the plan was to follow up as far as possible as per the protocol and perform a subset analysis. Withdrawals were aimed to be replaced if time permitted.

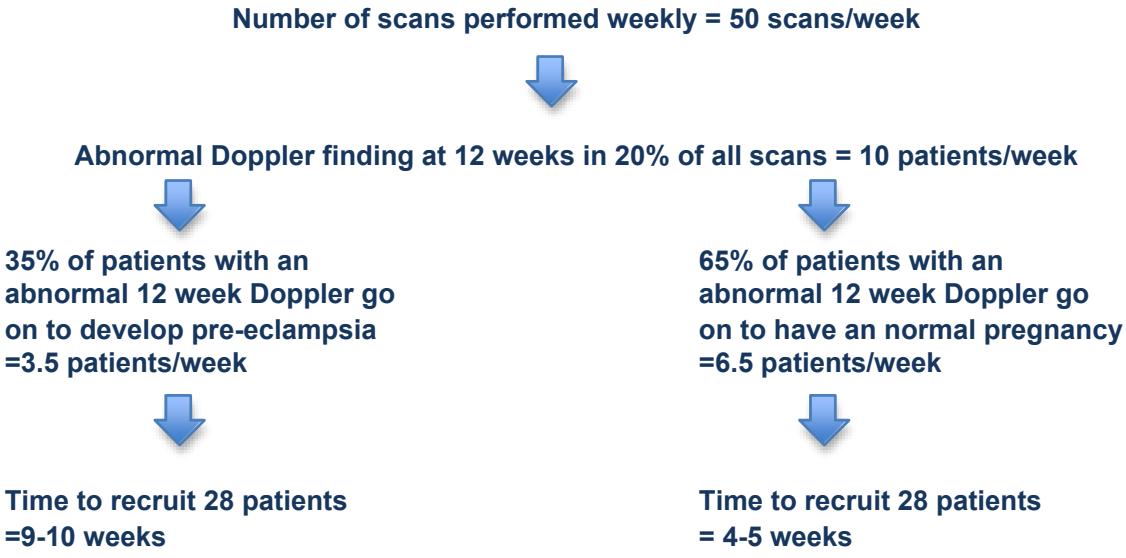
2.15.6 Sample size

This clinical study was designed as a pilot study to assess the effect of placental dysfunction with a postulated decrease in production of placental hormones and the subsequent impact on the primary endpoint of foetal adrenal enlargement. It was therefore apparent that a power calculation for this could only be performed retrospectively once the effect size is known. Instead I carried out a power calculation of sample size to detect a difference in maternal kisspeptin levels between normal pregnancies and those complicated by pre-eclampsia.

A case-control study [176] examining 3rd trimester kisspeptin levels in a group of women with pre-eclampsia and a group with normal pregnancies carried out statistical power calculations in order to detect a difference of 600pmol/l and an estimated standard deviation of 500, a power of 80%, and a difference detected at the 5% significance level (1–β

of 0.8 and α of 0.05 respectively). No statistically significant difference between the groups was found. However the study was powered to detect a difference in plasma kisspeptin concentrations of 600 pmol/l or greater, and therefore the relatively small number of pre-eclampsia patients (n=8) recruited into the study may not have allowed for the detection of more subtle differences in kisspeptin between the groups. In this study, statistical power calculations yield sample sizes of 25 controls and 25 experimental subjects in order to detect a difference between kisspeptin levels in the two groups of 400 pmol/l with an estimated standard deviation of 500pmol/l, 1–β of 0.8 and α of 0.05. Assuming a 10% dropout rate in each cohort, 28 patients would need to be recruited in each group. The time taken to recruit this sample number was based on figures obtained from a pilot study carried out in the Foetal Medicine Unit/Antenatal Clinic at the Royal London Hospital in 2011 (Mr Joseph Aquilina, unpublished).

Time to recruit study sample number



2.15.7 Primary endpoint

Correlation of placental kisspeptin (maternal kisspeptin levels) to foetal adrenal size.

2.15.8 Secondary endpoints

1. Gestational age at end of pregnancy and outcome of pregnancy (to include miscarriage, still birth, live birth).
2. Birth weight
3. Correlation of placental kisspeptin with other markers of placental function (hCG, PlGF)
4. Neonatal complications including respiratory distress syndrome, transient tachypnoea of the newborn, neonatal death.

2.15.9 Parameters that mark the end of the study

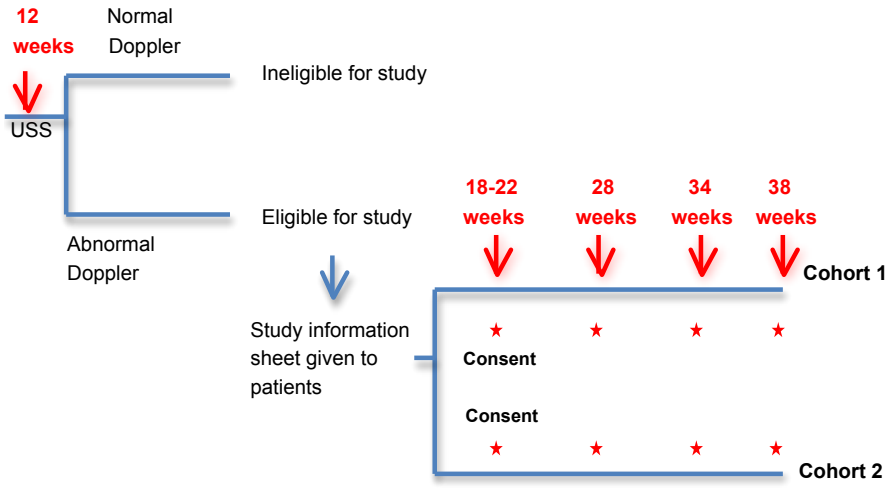
The outcome of infants of patients enrolled in the study was recorded.

Infants admitted for hospital care were followed up to discharge if this exceeded the planned duration of neonatal follow up for this study (8 weeks).

2.15.10 Clinical protocol

Clinical management was directed by, and at the discretion of, the obstetric and neonatal providers. Antenatal scans for the participants were performed in the Foetal Medicine Unit by two Obstetric Fellows (Dr Rebecca Allen and Dr Shemoon Marleen), under the guidance of the Lead for Foetal Medicine (Mr Joseph Aquilina). This collaborating team carrying out all the study scans were also providing clinical antenatal care for the patients enrolled in the study. They were therefore not blinded when performing scan measurements as this would have been unethical and compromised patient safety and care. Serial measurements of foetal adrenal size were performed at the time of the routine anomaly scan (18-22 weeks gestation, visit 1) and at 3 other time points: ~28, 34 and 38 weeks gestation (visits 2-4, respectively) (Fig. 14). Maternal plasma samples for kisspeptin (and other biomarkers, PlGF and hCG) were taken at the time of the first USS only (collection at ~20 weeks gestation as part of a separate study conducted by Dr Rebecca Allen) and subjects were followed up until the outcome of pregnancy was known.

Fig. 14. Antenatal Schedule

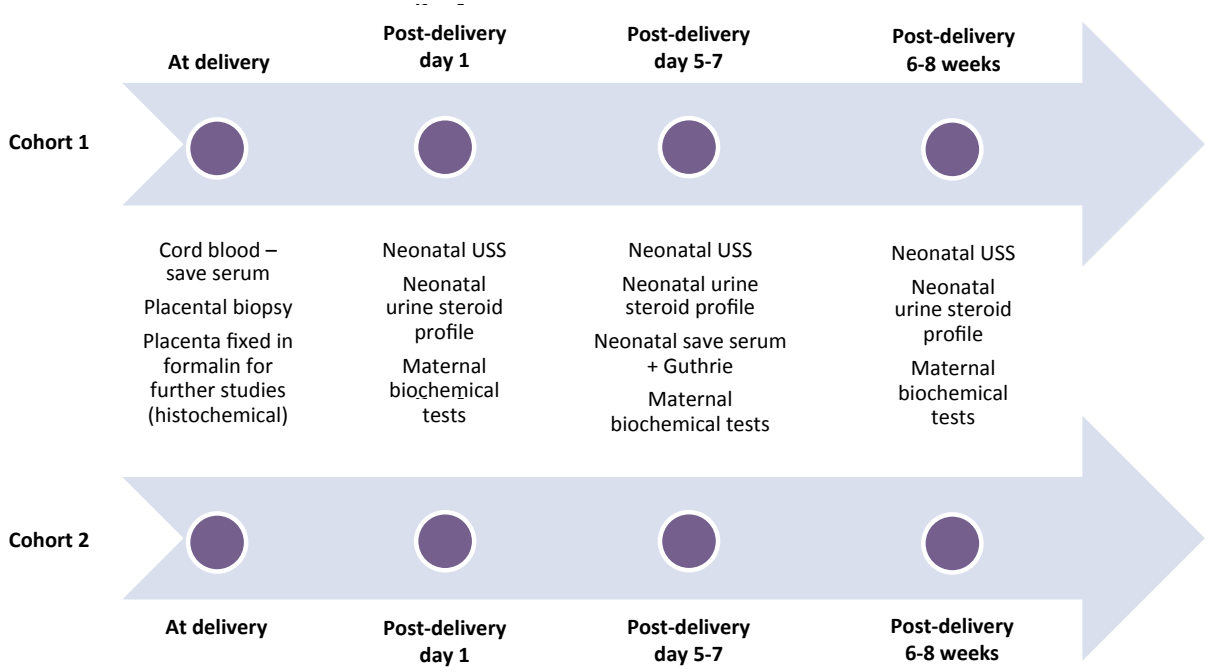


★ Antenatal USS FZ measurements and maternal biochemical tests
Cohort 1: Normal pregnancy (~65% of patients with an abnormal Doppler at 12 weeks)
Cohort 2: Pre-eclampsia (~35% of patients with an abnormal Doppler at 12 weeks)

As far as possible each delivery was attended by one of the study investigators (myself, Dr Muriel Meso, Miss Shezhan Elahi). At delivery, cord blood was obtained and stored. Placental punch biopsies were taken, and the placenta was sent for histology as part of a separate research study. With consent, each live infant of a mother enrolled in the study was followed up during the 1st week of life with a postnatal abdominal USS scan measuring the adrenal gland and urine collection as soon as possible after delivery while still in hospital, and again at ~5-7 days of life and with an USS, urine collection and a blood sample (Fig. 15). Routinely in the UK a Guthrie blood spot test is collected at 5-7 days of life. If consent is obtained, a whole blood sample was collected for storage at the same time that the Guthrie is performed by the study investigators (myself, Dr Muriel Meso, Miss Shezhan Elahi). The ultrasound scan and urine collection were repeated at 6-8 weeks of life. The postnatal scans were performed, using the same measurement technique as antenatal foetal scans, in collaboration with the Royal London Hospital Radiology department. The neonatal scans were all performed by one Consultant Paediatric Radiologist (Dr Kirsteen

Macdonald). Post-discharge, urine was collected at home overnight by parents the day before their follow up appointment and stored in the fridge, or collected on the day of the scan while in hospital. Urine collection involved a small plastic bag placed in the nappy and affixed gently to the skin. Neonates who are admitted to the neonatal unit or who require anything other than routine postnatal care were followed up until discharge. If the baby was too unwell to undergo an USS, this was performed later when it was clinically safe to do so.

Fig. 15. Postnatal schedule



Cohort 1: Infants of mothers who had uncomplicated pregnancies
Cohort 2: Infants of mothers who developed pre-eclampsia

For each infant, the following outcomes were recorded:

1. Outcome of pregnancy (stillbirth, miscarriage, live birth, neonatal death)

2. Gestation at birth

3. Birth weight

4. Complications of pregnancy / labour (e.g. intrauterine growth retardation)

5. Placental weight and morphology at delivery

6. Neonatal production of steroids (urine for steroid profile collected to be measured by LC-MS/MS at the Steroid Lab, Kings College Hospital)

7. Adverse neonatal outcomes (respiratory distress syndrome, transient tachypnoea of the newborn, necrotizing enterocolitis).

2.15.11 Kisspeptin RIA

Ten milliliters of blood were taken and collected into lithium-heparin tubes (LIP, Cambridge, UK) containing 5,000 kallikrein inhibitor units (0.2 ml) of aprotinin (Trasylol; Bayer, Newbury, UK) and stored on ice. After centrifugation, plasma was immediately separated and stored at −20°C until measurement of kisspeptin IR in all subjects. Samples were stored at -20°C for between 6 and 18 months prior to kisspeptin measurements. Plasma kisspeptin immunoreactivity (IR) was measured by collaborators using an established in-house radioimmunoassay at Imperial College London [177]. Antibody GQ2 was raised in a sheep immunized with synthetic human kisspeptin-54 (Bachem UK, Merseyside, UK) conjugated to BSA by glutaraldehyde and used at a final dilution of 1: 3,500,000. Bioactivity of kisspeptin-54 peptide was confirmed by stimulation of LH release in female mice. Vials of freeze-dried kisspeptin-54 were stored at -20OC and reconstituted in 0.9% (w/v) saline. The antibody cross reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with any other related human RF amide peptide, including prolactin releasing peptide, RF amide-related peptide (RFRP1), RFRP2, RFRP 3, neuropeptide FF, and neuropeptide AF. The 125I-kisspeptin-54 label was prepared using the iodogen method and purified by reverse-phase HPLC on a C18 column (Waters, Milford, MA) over a 15–45% 90-

min gradient of acetonitrile (AcN)/water/0.1% (v/v) trifluoroacetic acid (TFA). The specific activity of kisspeptin label was 56 Bq/fmol. The assay was performed in duplicate using dilutions of neat plasma in 0.7 ml of 0.06 M phosphate buffer (pH 7.2) containing 0.3% (w/v) BSA and incubated for 3 d at 4 C. Free and antibody-bound label were then separated by charcoal adsorption. The assay detected changes of 2 pmol/liter of plasma kisspeptin with a 95% confidence limit. The intraassay and interassay coefficients of variation were 8.3 and 10.2%, respectively.

2.15.12 Maternal placental biomarker measurements

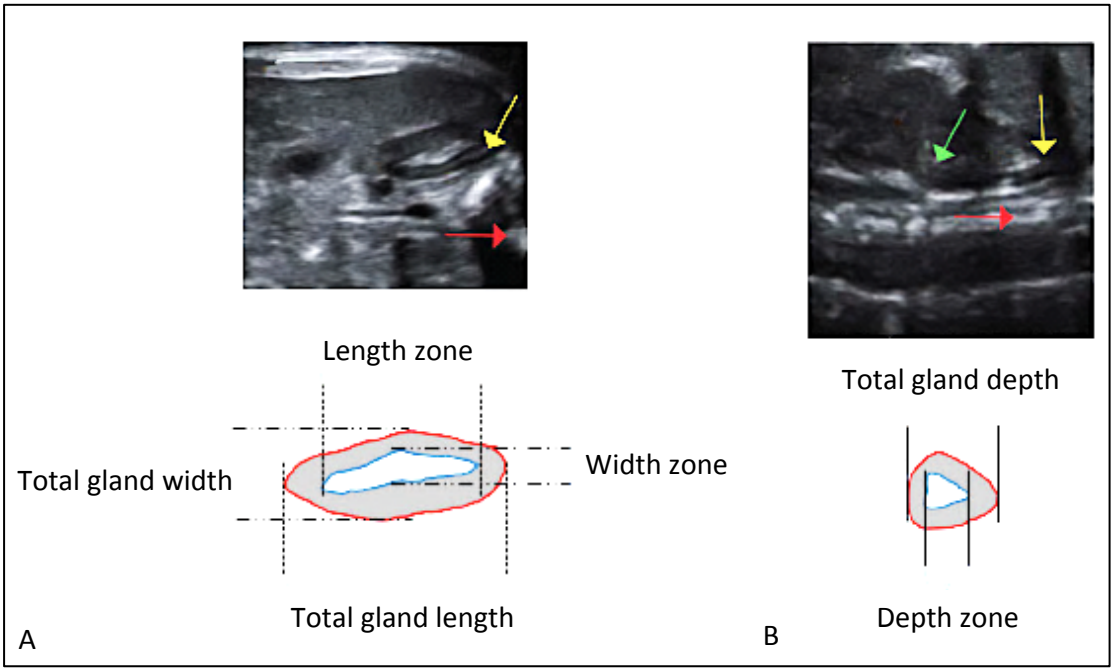
Ten mls of maternal blood was taken in gold top gel tubes with no additives. The blood samples were allowed to clot and then centrifuged with removal of serum into aliquots, which were stored at -80oC until analysed. 50µl samples were extracted for measurement of PlGF and 10 µl for β-hCG. Elecsys test kits from Cobas were used and analysed by a Roche Cobas e601. The lower limit for detection was 3pg/ml for PlGF, and <0.1iu/L for β-hCG. Tests of repeatability were performed to determine between batch imprecision and revealed a coefficient of variation of 3.89% for PlGF. These samples were analysed by the Biochemistry laboratory at the Royal London Hospital as part of a separate collaborative study conducted by Dr Rebecca Allen (Foetal Medicine Unit).

2.15.13 Adrenal ultrasound measurements

The gestational age was determined at the 12-week booking visit according to routine methods of ultrasound guided crown-rump length assessment. Following this serial 3-

dimensional transabdominal ultrasound measurements took place at approximately 20, 28, 34 and 38 weeks' gestation. Each scan was performed transabdominally by one of the two collaborating senior foetal medicine fellows (Dr Rebecca Allen, Dr Shemoon Marleen). Assessment of the foetal adrenal glands was done according to the method described by Turan [178] along with foetal biometry, using the Voluson E8 systems (Voluson Expert; General Electric Healthcare Austria GmBH & Co; Zipf; Austria) equipped with a 4-8 MHz array transducer. FA depth, width and length parameters were obtained. For optimal visualisation the foetal adrenal gland closest to the probe was selected for analysis and subsequently assessed in the transverse, coronal and sagittal plane to obtain the gland length, width and depth (Fig. 16a, b). Based on these parameters a 3D-ultrasound model for the adrenal gland volume was obtained using the VOCAL (Virtual Organ Computer-Aided Analysis, 4D View; General Electric Medical System, Milwaukee, WI) software package. For accuracy each Fellow reviewed all images separately before both calculated the volume independently. For each participant the abdominal circumference (AC), femur length (FL) and biparietal diameter (BPD) were also measured. Based on these parameters the estimated foetal weight (EFW) was calculated for all gestational ages >20 weeks using the GE ViewPoint 6™ software (ViewPoint Bildverarbeitung GmbH; Wessling, Germany). Women were followed until the outcome of their pregnancy was known. Adrenal volume data were missing in several patients who failed to attend USS appointments (Table 7).

Fig. 16a: Methodology of measurement of the whole adrenal gland



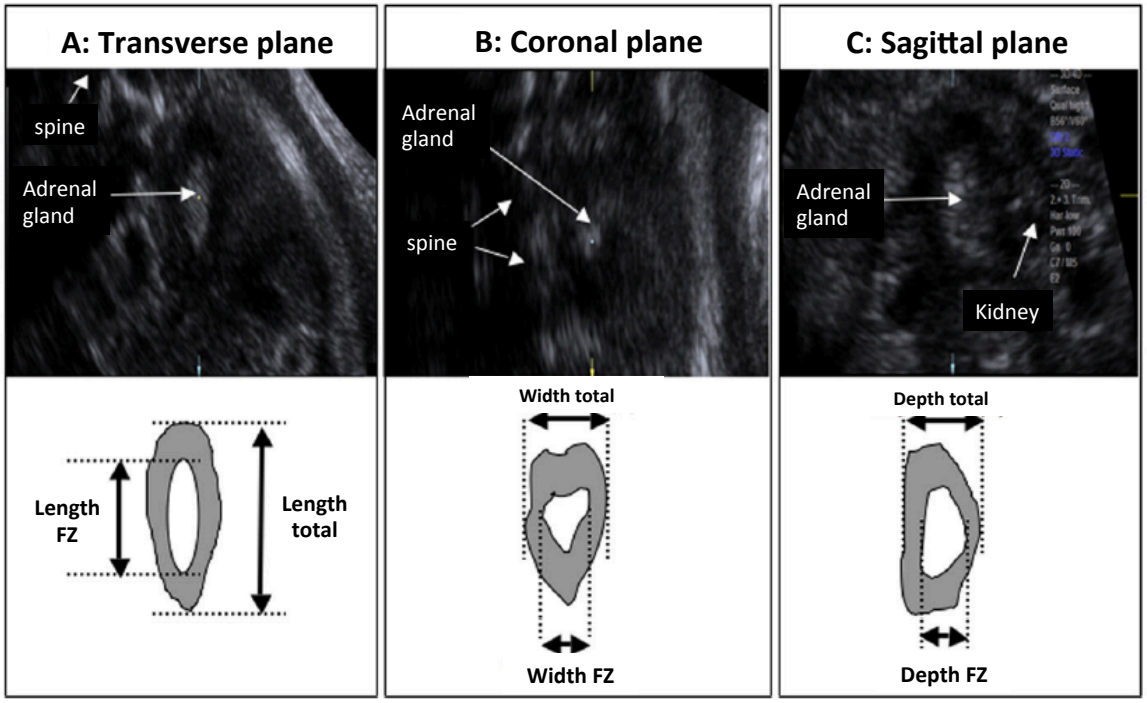
1. Transverse measurements. *Yellow arrow*, adrenal gland; *red arrow*, spine.

2. Sagittal measurements. *Yellow arrow*, adrenal gland; *red arrow*, spine; *green arrow*, kidney.

3. Transverse measurements to obtain foetal adrenal length were also obtained (not shown).

(Adapated from Turan et al., 2011).[178]

Fig. 16b: Methodology of measurement of the foetal zone



Ultrasound image of the A, transverse, B, coronal and C, sagittal plane, and corresponding schematic images of adrenal gland and foetal zone were demonstrated. The adrenal gland, kidney, and spine are marked by arrows. (Adapated from Turan et al., 2011).[178]

2.15.14 Data handling and storage

Data was stored in accordance with local and national data protection regulations. The Investigators had responsibility to ensure that patient anonymity is protected and maintained. Information with regards to study patients was kept confidential and managed in accordance with the Data Protection Act, NHS Caldicott Guardian, The Research Governance Framework for Health and Social Care and Research Ethics Committee Approval.

Tissue samples from HDBR and Kuopio were annonymised with all patient identifiable details removed. The data was stored on password-protected databases on computers at the William Harvey Research Institute.

Data collected from the clinical study was pseudo-anonymised. Patient details were coded and data separated on input. Identifiable information collected from the subjects included the hospital number, name and date of birth. This information was required in order to process samples at the laboratory at the Royal London Hospital. Participants received a unique identifying code and these codes, the key to the corresponding identifiable information, and the personal information itself was stored on separate password protected databases at the WHRI, QMUL. Only the PI of the study (Dr Helen Storr) had further access to the database containing identifiable information and the key. The other study investigators only had access to the database with the corresponding codes and data was inputted and then analysed in this format. No patient identifiable details will be transferred outside the European Union and participants had the right to revoke their authorisation for the use of their information and data. Patient information was anonymised with regards to publications relating to this study.

2.15.15 Data analysis and statistics

In-vitro data were evaluated using a paired two-tailed Student's t test or one-way ANOVA followed by a post-hoc Tukey comparisons test (GraphPad Prism 6, San Diego, CA). All experiments were performed in triplicate and represent 3 or 4 independent experiments; error bars depict the standard deviation of each individual experiment. Non-parametric continuous variables (FAV and kisspeptin levels) were analysed by a Kruskal–Wallis test with

Dunn-Bonferroni post hoc multiple comparison test correction. Continuous parametric variables (FAV in male and female foetuses) were compared using student t-test. R-values are Pearson's correlation coefficient (SPSS V.23 Armonk, New York, USA: IBM Corp). Statistical analysis of the clinical data was carried out by Dr Sumana Chatterjee (Clinical Fellow, Paediatric Endocrinology). P values <0.05 were statistically significant.

3 Results: Expression of Kiss1R in the developing human foetal adrenal cortex

3.1 Aims

The main aim of this work was to obtain a detailed description of the expression of Kiss1R within the developing HFA cortex. To date, only one previous study has examined foetal adrenocortical expression of Kiss1R [165]. Quantitative reverse-transcriptase PCR (qPCR) was used to compare mRNA levels for *Kiss1R* between foetal and adult adrenal glands. *Kiss1R* was reported to be significantly higher (50-fold) in HFA than in human adult adrenals. In addition, immunohistochemical studies carried out by this group demonstrated that the Kiss1R protein is predominantly expressed in the outer DZ and TZ of HFA cortex from 14-36 weeks gestation (12wpc – 34wpc).

Early expression of Kiss1R in the developing HFA prior to 14 weeks gestation (12 wpc) has not previously been described. In this study, co-localisation studies using markers of zonation to further characterise the spatial expression of Kiss1R, and tissue from early gestation (8wpc, 10 weeks gestation) to term (38wpc, 40 weeks gestation) were studied to examine the temporal expression of Kiss1R. CD56 (neural cell adhesion molecule) is an adhesion molecule that belongs to the Ig superfamily of adhesion molecules and has roles in axonal growth, migration, and guidance. CD56 expression was originally thought to be confined to neuronal and neuroendocrine tissues, however expression has been noted in other tissues, most notably endocrine organs including human adrenocortical cells [179]. Interestingly, the adult adrenal gland expresses CD56 primarily in the zona glomerulosa [179]. Late in gestation, the DZ, which also is immediately beneath the capsule, is analogous to the zona glomerulosa in that it begins to express steroidogenic enzymes and produce aldosterone. CD56 expression has previously been observed by immunohistochemistry on DZ cells [17]. CD56 mRNA expression has also been detected among DZ cells but not selected sections of FZ cells isolated by laser capture microdissection [17]. These findings indicate that

CD56 expression can be used as a marker of DZ cells. Search for a cell surface marker for FZ cells led to the LDL (low density lipoprotein) receptor, as circulating LDL cholesterol is a major precursor for adrenal steroid synthesis [180]. As the cells of the FZ are steroidogenic beginning in early gestation, whereas those of the DZ are not, it can be predicted that the LDL receptor might exhibit zonal expression in the HFA. Previous studies using immunofluorescence have shown far greater expression of the LDL receptor on cells of the FZ than the DZ. DZ cells would not be expected to express the LDL receptor early in gestation given their lack of steroidogenic activity until close to term, however the evolution to a steroid-producing phenotype in the DZ is a limitation of using this as a marker of FZ cells. SULT2A1, which converts DHEA to DHEAS, has been shown to localize to the TZ and FZ, but not to the DZ [20]. Therefore, in these studies CD56 was chosen as a marker of DZ/TZ cells and SULT2A1 was chosen as a marker of FZ cells. Steroidogenic factor-1 (SF1) a key transcriptional activator of numerous genes involved in steroidogenesis, is expressed in almost all adrenocortical cells in the DZ, TZ, and FZ [181]. SF1 is expressed in the HFA from its earliest stages of adrenal development, initially in the adrenogonadal precursors, and subsequently in both the FZ and DZ of the gland [182]. SF1 was therefore used in studies as a spatio-temporal marker of foetal adrenocortical cells.

3.2 Morphological study of the developing human foetal adrenal

H&E staining of HFA sections demonstrated the morphology and remarkable growth of the gland (seen in comparative images from 14 wpc – 38 wpc, 16 – 40 weeks gestation) (Fig. 17). The large size of the foetal adrenal is evident when compared to the foetal kidney (Fig. 17 A, arrow). The zonal architecture is evident in Fig. 17 and Fig. 18. The cytoplasm of cells in the outer definitive/transitional zones (DZ/TZ) exhibit some basophilia; in combination with the

closely packed nuclei, this gives this part of the gland a blue appearance, in contrast to the eosinophilic pink staining of the inner foetal zone (FZ) (Fig. 18).

An extensive body of evidence delineates essential roles for SF1 in regulating adrenocortical differentiation and function [20]. Immunofluorescence studies to localise steroiodogenic cells in the foetal adrenal were carried out (Fig. 19, HFA at 11wpc shown). Localization of SF1-positive steroidogenic cells (green) was demonstrated, as previously described [182], throughout the foetal adrenal cortex (panel A). No immunoreactivity was detected in the negative controls where the primary antibody (SF1) was omitted (panel B, SF1-con).

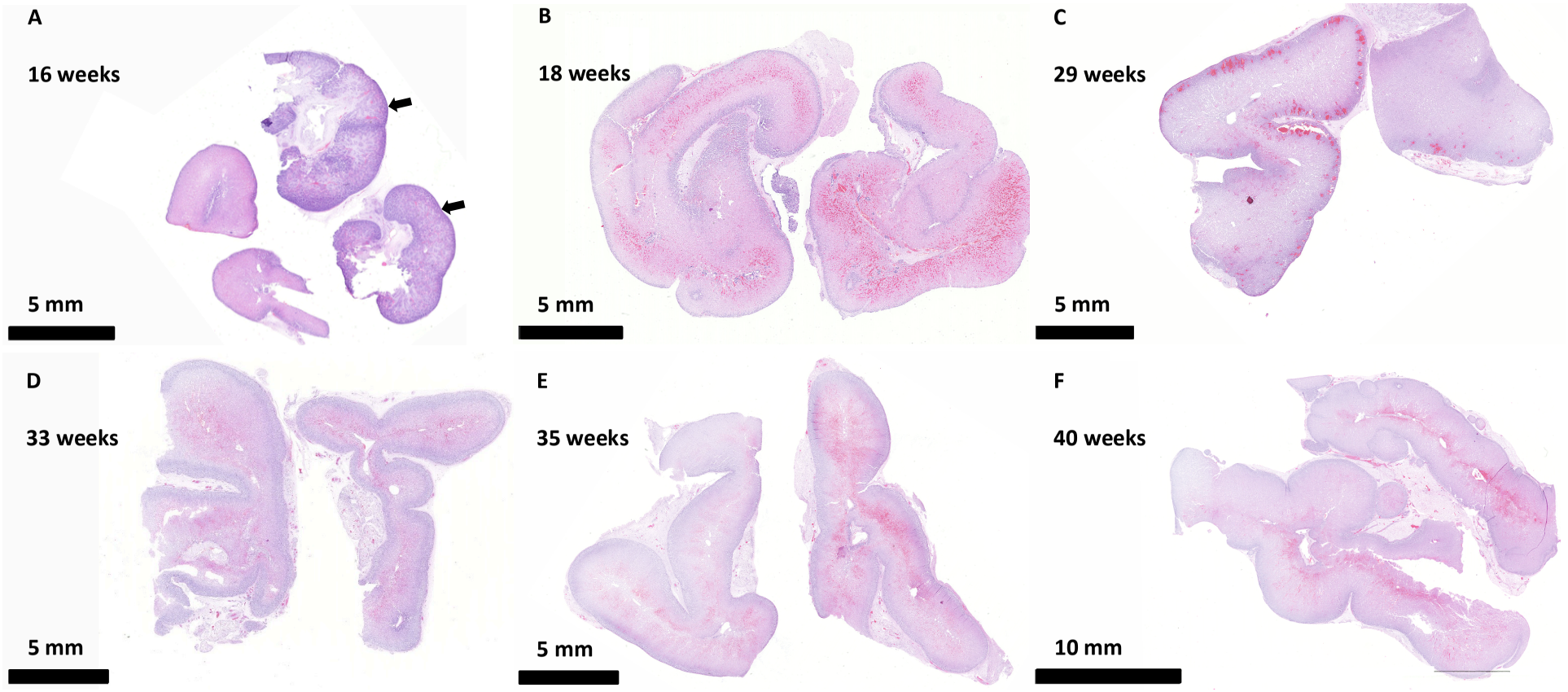


Fig. 17. H&E staining showing the morphology of the developing human foetal adrenal (14 – 38wpc, 16 – 40 weeks gestation; 2 adrenals per time point). The large size of the gland is demonstrated at 16 weeks in comparison to the foetal kidney (arrow) (A).

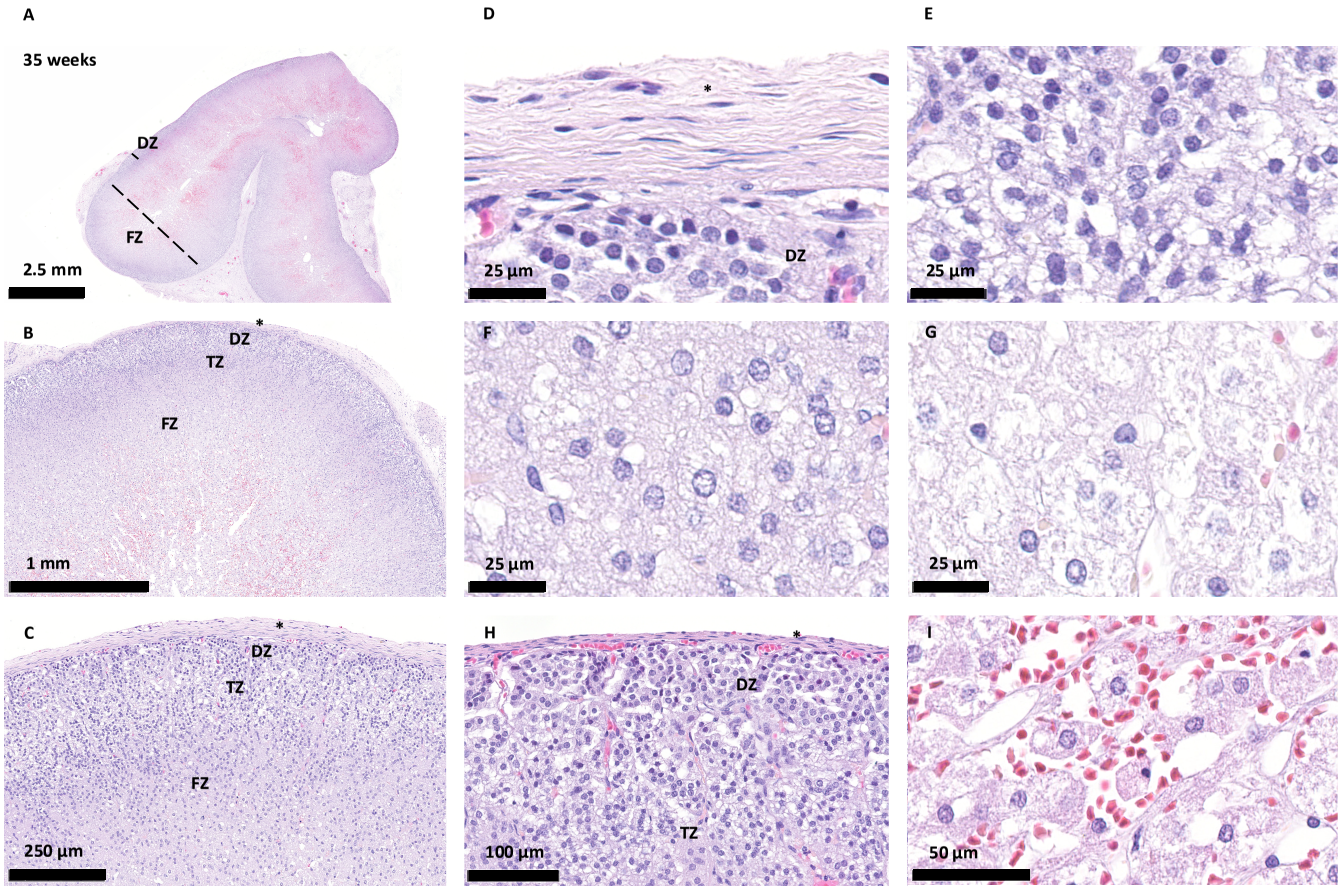


Fig. 18. H&E staining of the human foetal adrenal gland at 35 weeks gestation. A thin capsule (*) surrounds the outer definitive zone (DZ) (A-D). The DZ of the cortex is the most superficial layer with closely packed, darker stained cells (A-D, E, H). The deeper layer with the more eosinophilic appearance is the foetal zone (FZ) (A-C, G). The transitional zone (TZ) lies between the outer DZ and inner FZ (B-C, F, H). The cortical cells are arranged in cords with vascular sinusoids between them (H, I).

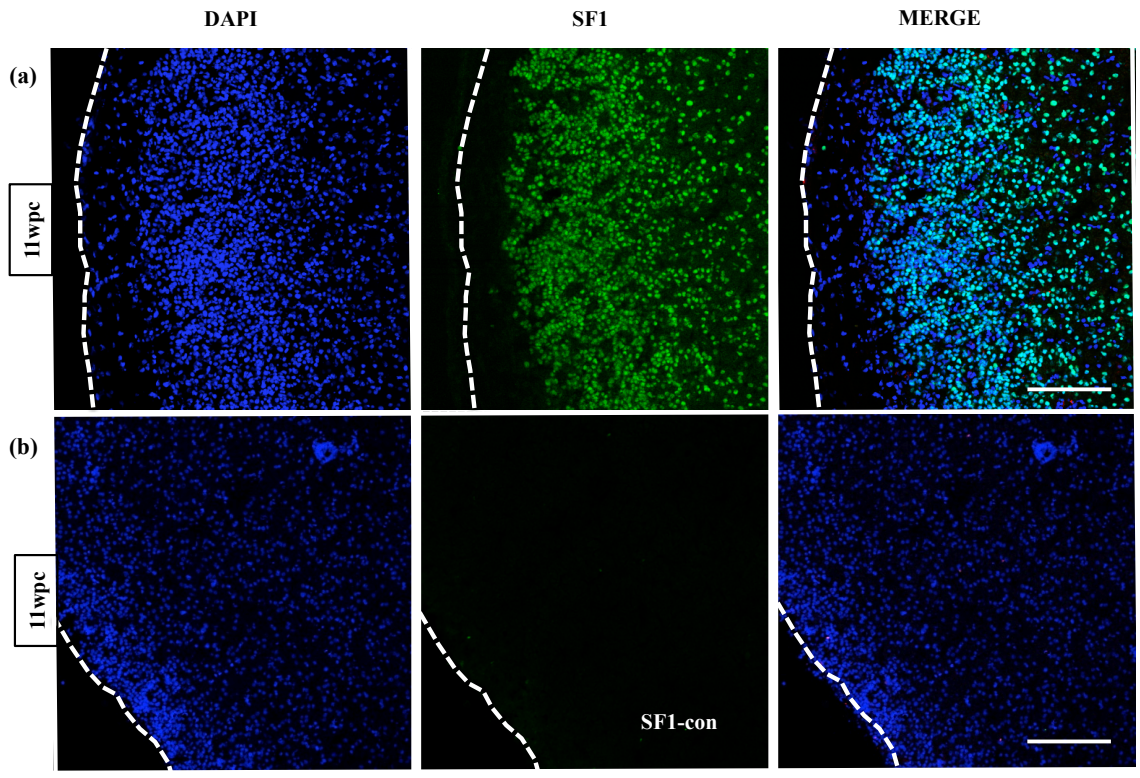


Fig. 19. Immunofluorescence studies of 11wpc HFA. Localization of SF1-positive steroidogenic cells (green) is demonstrated throughout the cortex using anti-SF1 antibody, (panel a). No immunoreactivity detected in the negative controls where the primary antibody (SF1) was omitted (panel b, SF1-con). The adrenal cortex is surrounded by an outer mesenchymal capsule (dashed line). Scale bar: 100µm.

3.3 Kiss1R is expressed from as early as 10 weeks gestation (8 wpc) to term (38 wpc, 40 weeks gestation) throughout the human foetal adrenal cortex in all zones (DZ, TZ and FZ).

The temporal and spatial expression of Kiss1R in the foetal adrenal cortex was characterised by performing immunofluorescence studies on a series of tissue ranging from 10 to 40 weeks gestation (8 – 38 wpc).

3.3.1 Kiss1R is expressed in the first trimester from 10 weeks gestation (8pcw) in the developing adrenal cortex.

Kiss1R positive immunoreactivity in the HFA cortex was shown for the first time to be present from as early as 10 weeks gestation (8wpc). Steroidogenic cells are identified by SF1-immunoreactivity throughout the HFA and there is co-localization of SF1 (green) and Kiss1R (red) (Fig. 20, panels a and b).

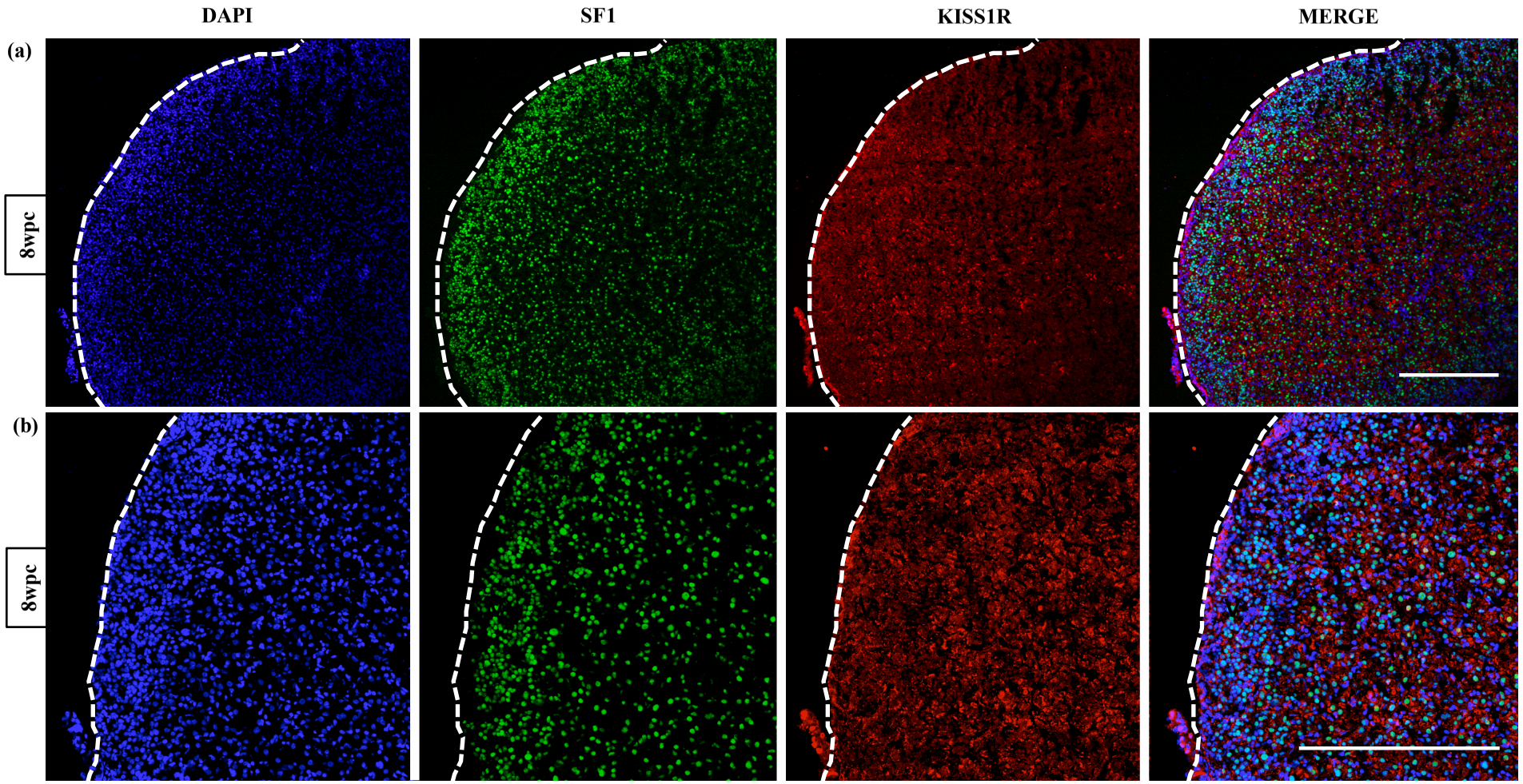


Fig. 20. Immunofluorescence studies of the human foetal adrenal at 8 wpc. Localisation of Kiss1R (red using anti-Kiss1R antibody (Alomone) and SF1 (green) using anti-SF1 antibody (Invitrogen). The adrenal cortex is surrounded by an outer mesenchymal capsule (dashed line). Scale bar: 100µm.

3.3.2 Kiss1R is expressed in all zones of the foetal adrenal cortex

Kiss1R positive immunoreactivity in the HFA cortex was shown for the first time to be present throughout the adrenal cortex, with expression in the inner FZ at all gestational stages examined (Fig. 21, high power of the FZ at 11 wpc) as well as the outer DZ and TZ (Fig. 22 a-c, 11wpc shown). This expression was specific, with no Kiss1R immunopositivity seen in the negative control where Kiss1R was preincubated with its peptide antigen (Fig. 23, panel b-c, Kiss1R-con). Co-localisation studies using markers of zonation for the outer DZ/TZ (CD56) and inner FZ (SULT2A1) revealed clear expression of Kiss1R in all zones of the developing cortex (Fig. 24).

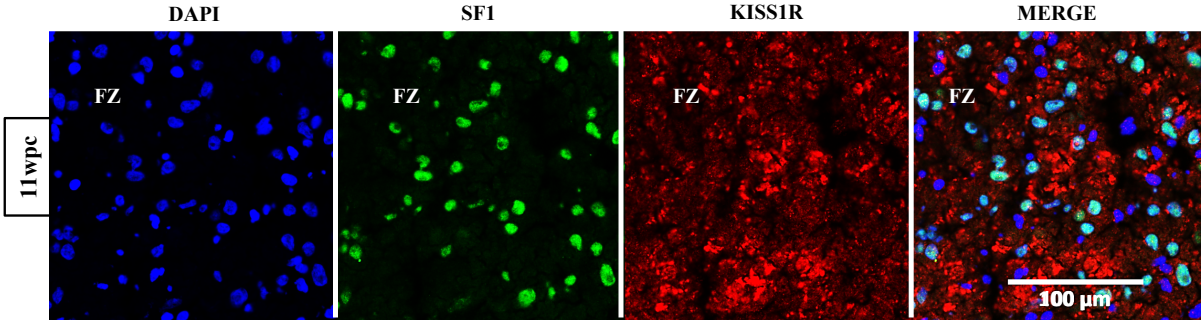


Fig. 21. Immunofluorescence studies of the human foetal adrenal (11 wpc shown). Localisation of Kiss1R (red) using anti-Kiss1R anitibody (Alomone) is seen in the inner FZ. Scale bar: 100µm.

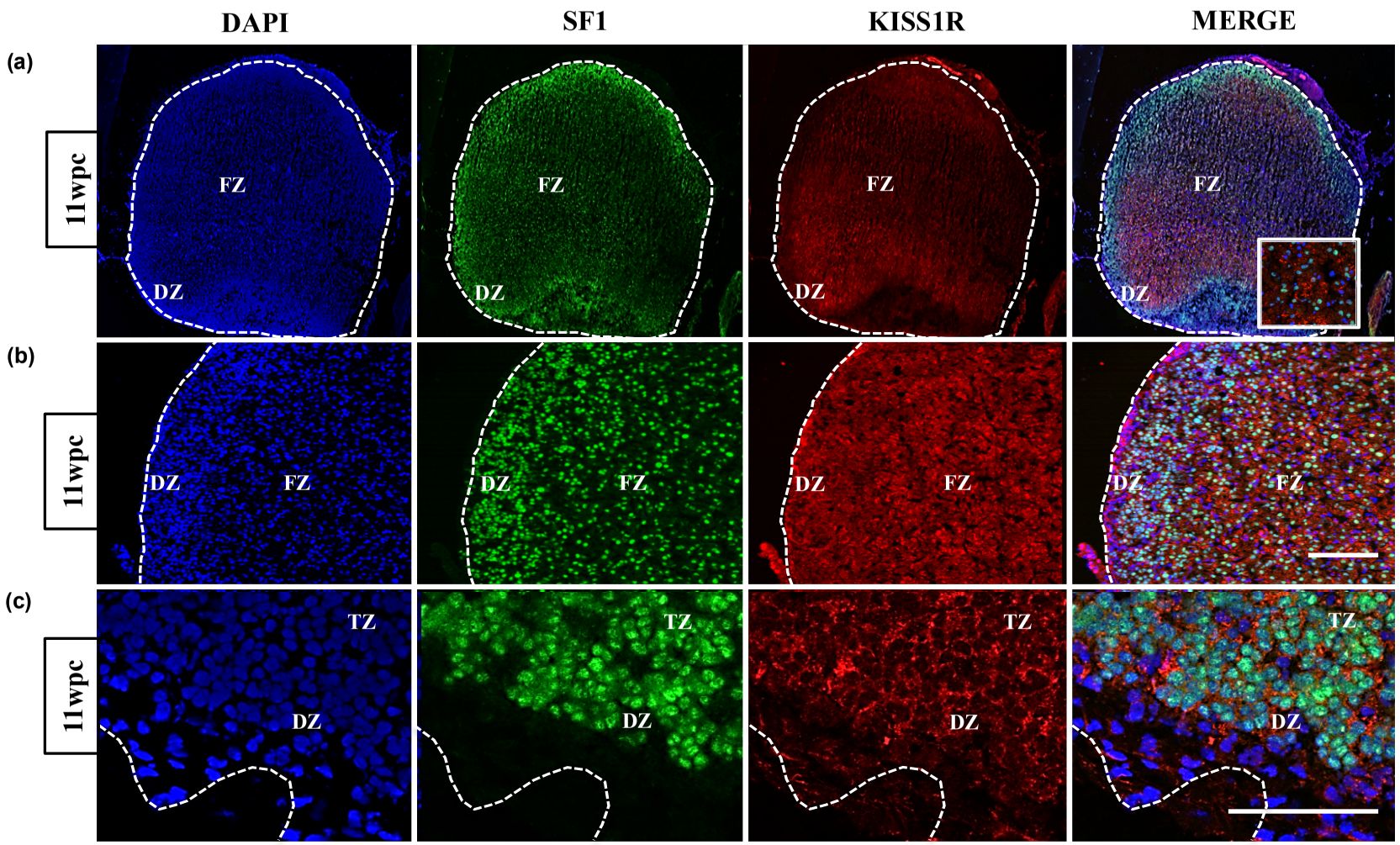
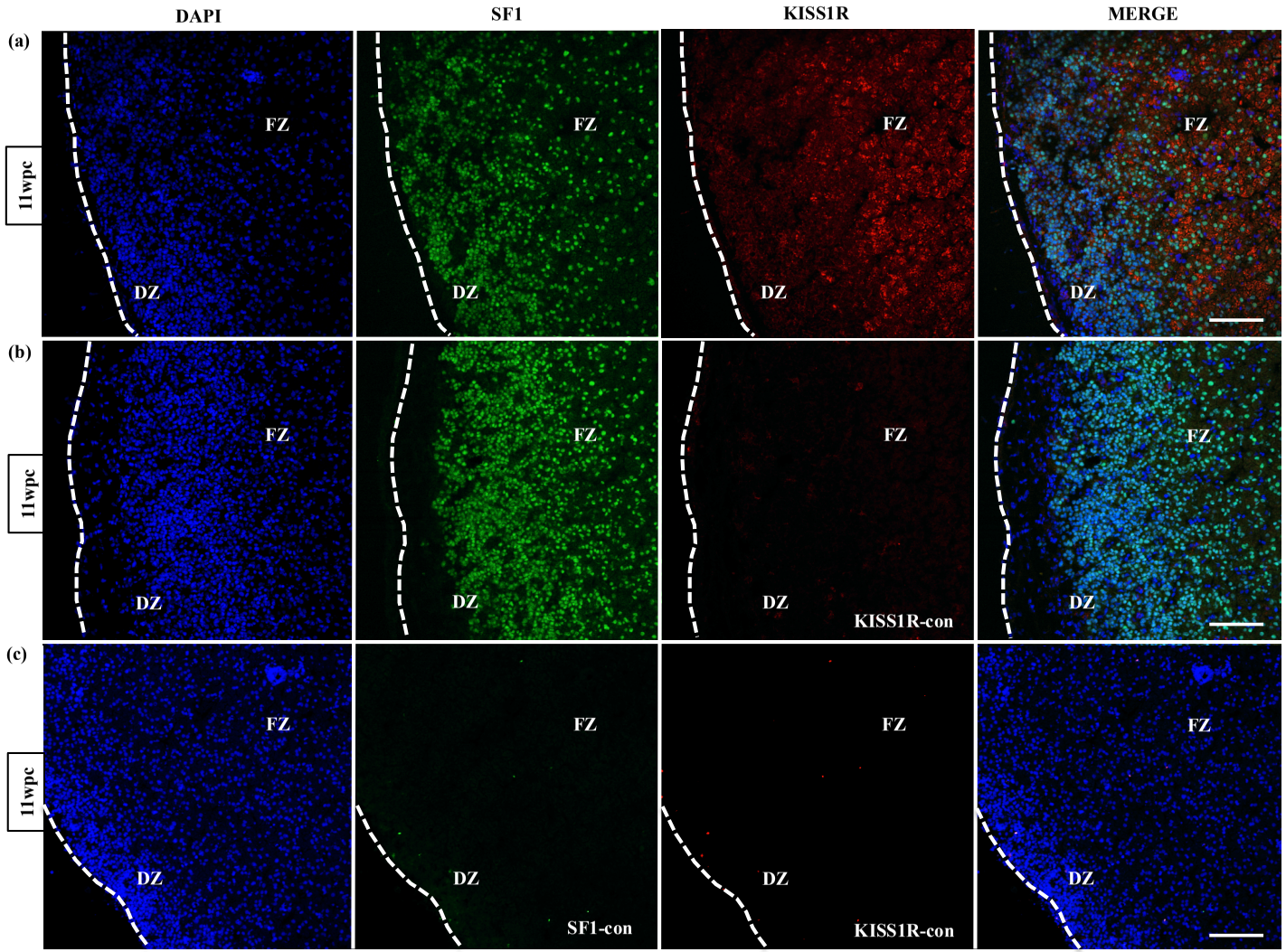
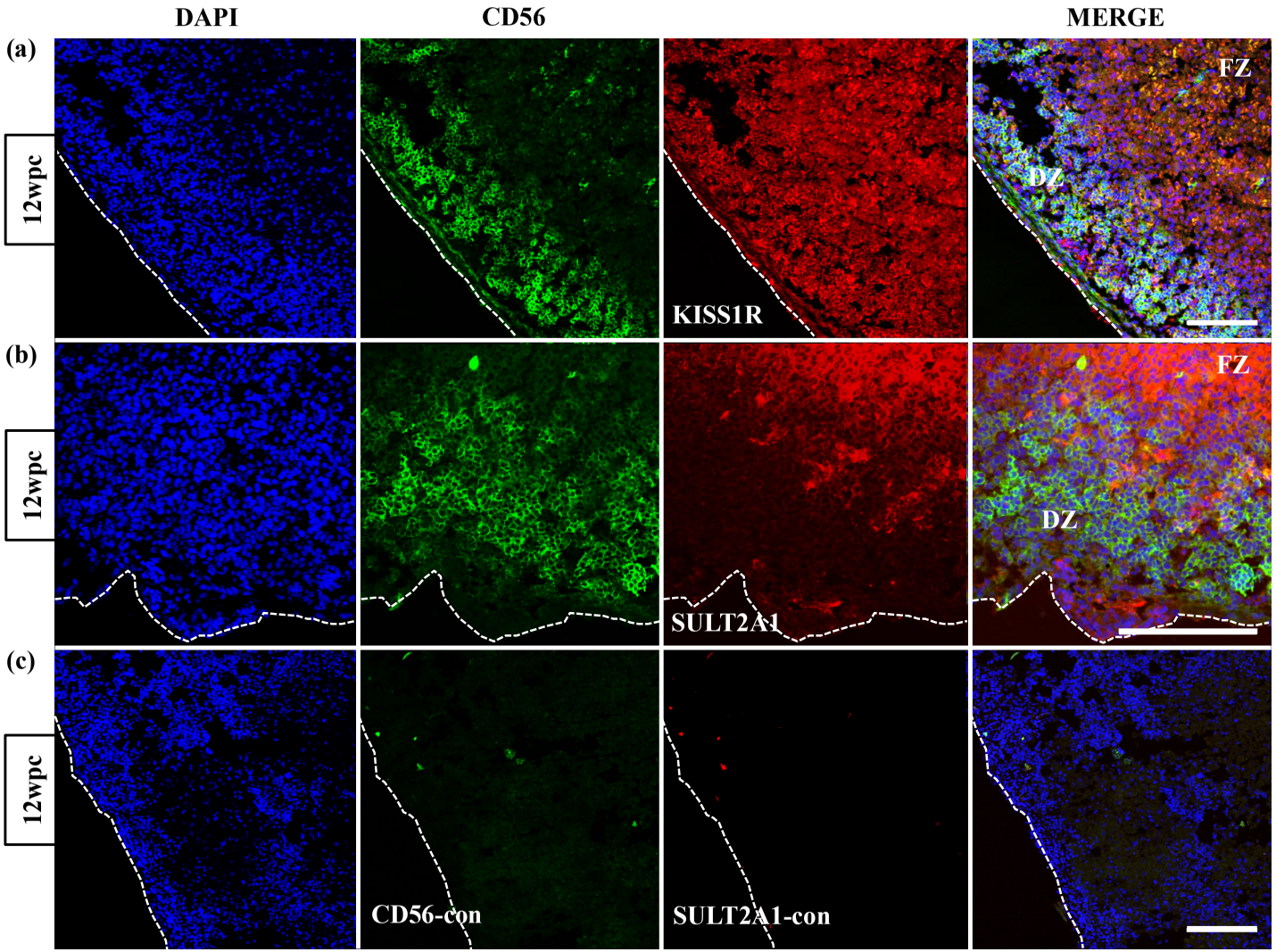


Fig. 22. Immunofluorescence studies of the human foetal adrenal (11 wpc shown). Localisation of Kiss1R (red) using anti-Kiss1R anitibody throughout the inner FZ, as well as the outer DZ/TZ. The adrenal cortex is surrounded by an outer capsule (dashed line). Scale bar: 100µm



Localisation of Kiss1R (red) using anti-Kiss1R anitibody (Alomone) throughout the inner FZ, as well as the outer DZ/TZ (panel a). Localisation of SF1 (green) is seen throughout the cortex (panels a-b). No immunoreactivity detected in the negative control with antigen (Kiss1R) preincubation, demonstrating specificity of the Kiss1R antibody (panels b-c, Kiss1R-con). No immuno-reactivity detected in the negative control for SF1 (primary antibody omitted) (panel c). Scale bar: 100µm.

Fig. 23. Immunofluorescence studies of the human foetal adrenal (11 wpc shown).



Localisation of CD56 (green) in the outer DZ/TZ (panels a-b) using anti-CD56 (Invitrogen). Localisation of SULT2A1 (red) in the inner FZ (panel b) using anti-SULT2A1 (Abcam). Localisation of Kiss1R (red) throughout the cortex (FZ and DZ) (panel a) using anti-Kiss1R antibody (Alomone Labs). No immuno-reactivity detected in the negative controls in which the primary antibody was omitted (panel c, CD56-con, SULT2A1-con). The foetal adrenal cortex is surrounded by an outer capsule (dashed line). Scale bar: 100µm

Fig. 24. Immunofluorescence co-localisation studies (12 wpc shown).

3.3.3 Kiss1R expression persists in all zones of the foetal adrenal cortex throughout early gestation to term (38 wpc, 40 weeks gestation).

Kiss1R positive immunoreactivity in the HFA cortex was shown for the first time after 36 weeks gestation (34 wpc), with expression in the DZ and TZ as well as the inner FZ (Fig. 26, 38 wpc (40 weeks gestation shown). This expression was specific, with no Kiss1R immunopositivity seen in the negative control where Kiss1R was preincubated with its peptide antigen (Fig. 25, Kiss1R-con, Fig. 27 panel e, Kiss1R-con). Kiss1R expression throughout all zones of the foetal adrenal cortex is therefore present from as early as 8 wpc (10 weeks gestation) and persists during mid-gestation in the 2nd and late-gestation in the 3rd trimester to term (38 wpc, 40 weeks gestation) (Fig. 26).

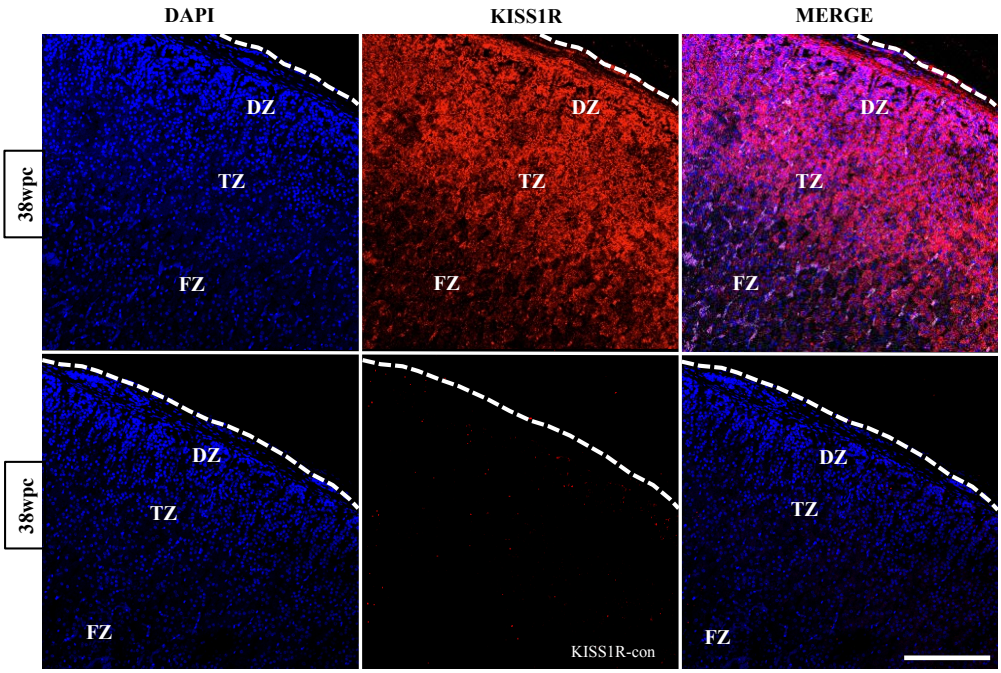


Fig. 25. Immunofluorescence studies of the human foetal adrenal at 38 wpc (40 weeks gestation). Localisation of Kiss1R (red) using anti-Kiss1R antibody (Alomone) in the DZ, TZ and throughout the FZ. No immunoreactivity detected in the negative control with antigen (Kiss1R) pre-incubation, demonstrating specificity of the Kiss1R antibody. The adrenal cortex is surrounded by an outer capsule (dashed line). Scale bar: 100µm.

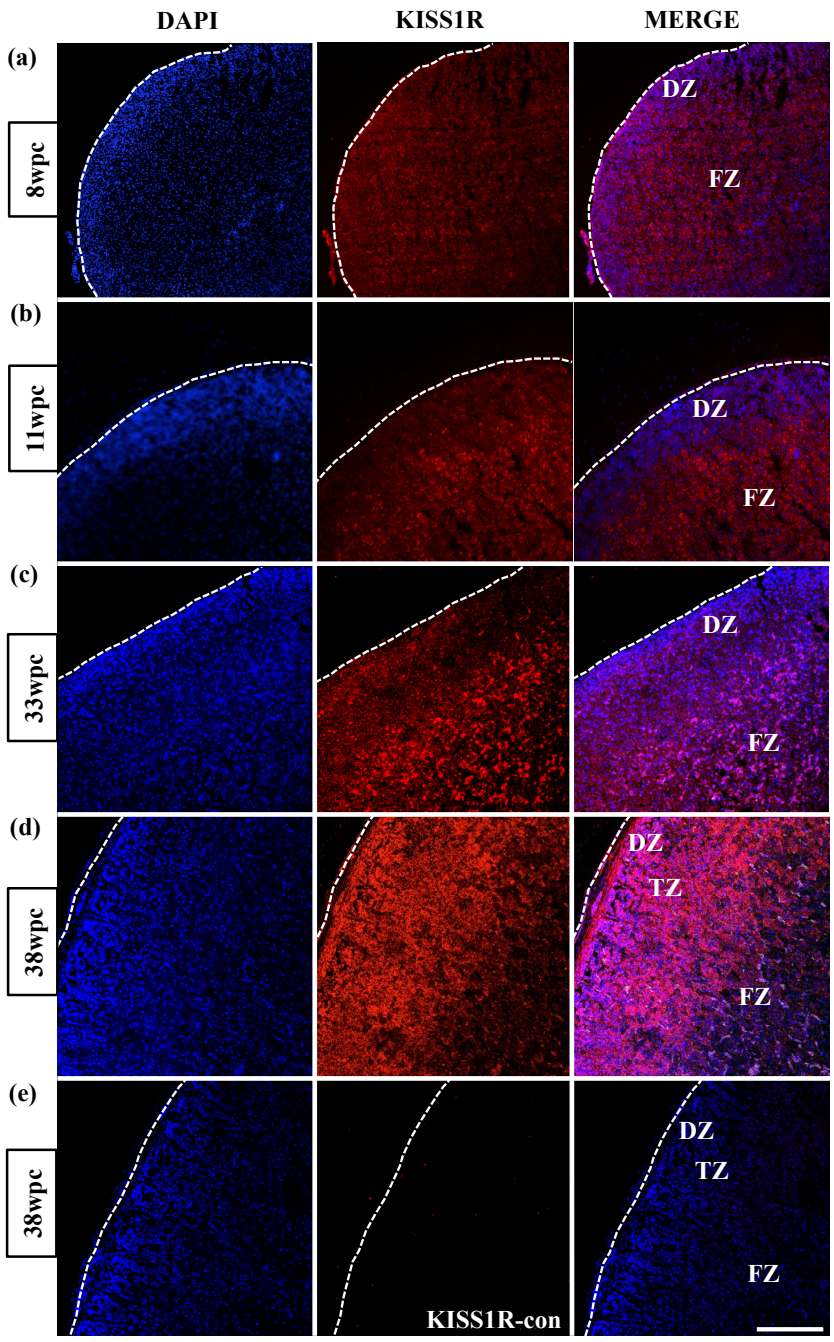


Fig. 26. Immunofluorescence studies of the human foetal adrenal at early, mid and late gestation (panels a-d, 8 wpc, 11 wpc, 33 wpc 38 wpc shown). Localisation of Kiss1R (red) using anti-Kiss1R antibody throughout the adrenal cortex at all gestational ages examined. No immunoreactivity detected in the negative control with antigen (Kiss1R) pre-incubation demonstrating specificity of the Kiss1R antibody (panel e, Kiss1R-con). The adrenal cortex is surrounded by an outer capsule (dashed line). Scale bar: 100µm.

3.3.4 NR-*in situ* hybridisation studies of *Kiss1R* expression in the human foetal adrenal confirm localisation to all zones of the foetal adrenal cortex).

To further support my novel finding of Kiss1R expression throughout the adrenal cortex, the mRNA expression pattern of genes encoding *Kiss1R* was determined using non-radioactive *in situ* hybridization (NR-ISH). This approach was chosen as I failed to obtain reproducible results on tissue sections with another commercially available antibody raised against Kiss1R, used in a previous study by Nakamura et al (immunohistochemical studies not shown). I therefore designed and tested antisense RNA probes for this gene, and confirmed they specifically detect *Kiss1R* expression in previously documented embryonic locations (Fig. 27, panels h, i). *In situ* hybridization was performed on sections obtained from human embryos and assayed with antisense (AS) and sense (S) probes. In the foetal adrenal tissues analysed (Fig. 27, panels a, c, e, f) *Kiss1R* transcripts localised to the DZ, TZ and FZ, in keeping with previous immunofluorescence studies. No specific signal was obtained when sections were treated with the sense probes (Fig. 28, panels b, d). *Kiss1R* expression was seen in foetal spinal cord and in the outer syncytiotrophoblasts of first trimester placental villi (Fig. 27, panel i) in keeping with numerous published studies [119,122,161,183]. Foetal lung tissue was used as a negative control (Fig. 27, panel g).

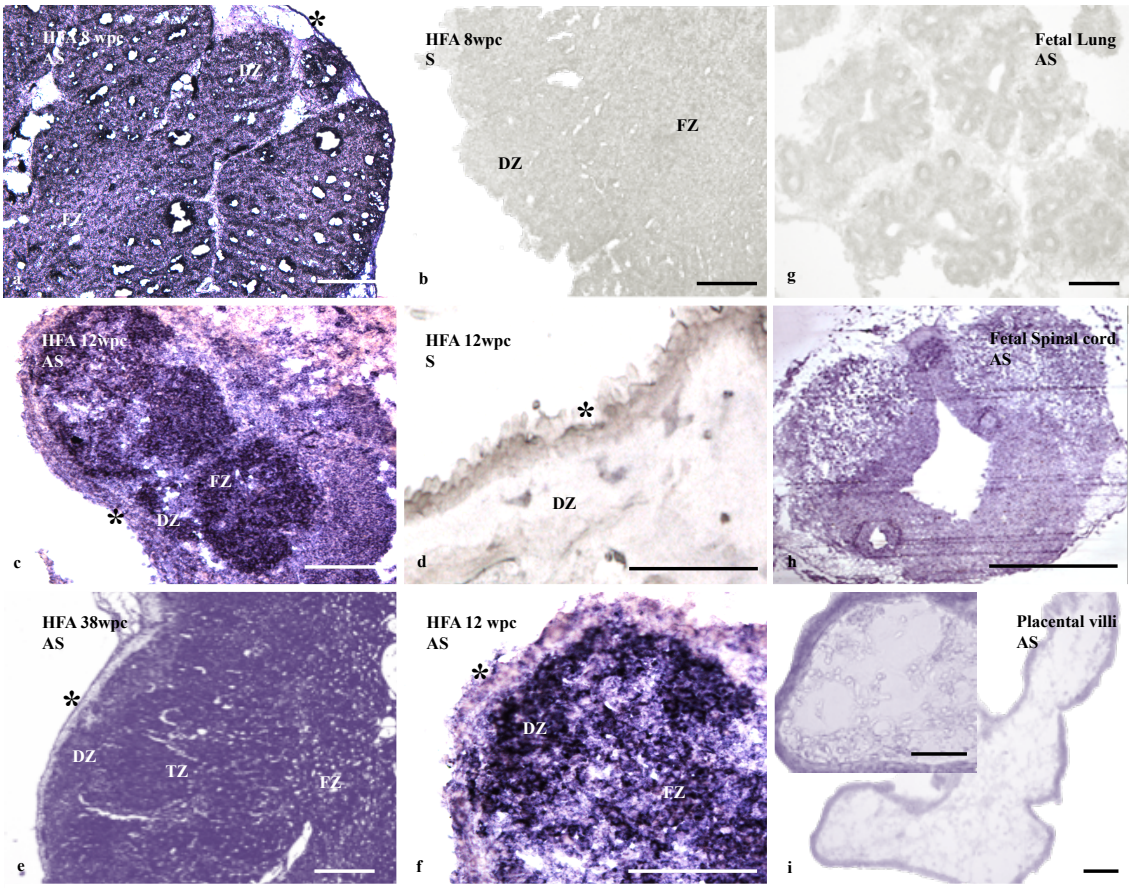


Fig. 27. NR-ISH studies of expression of *Kiss1R* transcript in human foetal tissue.

***Kiss1R* is localized throughout the developing human adrenal cortex (panels a,c,d,e). The capsule (*) is unstained. At all ages examined the adrenocortical cells were strongly positive for *Kiss1R* transcript (panel a, 8wpc, panel c, f 12wpc, panel e, 38 wpc shown). Panel g is foetal lung tissue (12wpc) used as a negative control. Panel h is foetal spinal cord (12wpc) and panel i is first trimester placental villi tissue (12 wpc) used as positive controls. Sections incubated with *Kiss1R* sense probe (panels b, HFA 8wpc, panel d, HFA 12 wpc) did not show any specific staining. Scale bars: 100µm.**

3.3.5 Quantitative assessment of Kiss1R in the developing human adrenal cortex.

A quantitative assessment of Kiss1R in the HFA was compared to expression in the placenta, where it is highly expressed. Kiss1R expression in the foetal adrenal was also compared to

that of SULT2A1. SULT2A1 (sulphotransferase) is highly expressed in the FZ of the developing adrenal cortex, and the ZF of the adult adrenal, where it converts DHEA to DHEAS. Quantitative reverse-transcriptase PCR (qPCR) performed on 1st and 2nd trimester HFA cDNA (8-10wpc, 11-14wpc, 15-20wpc) showed an increase in both *Kiss1R* (Fig. 28A) and *SULT2A1* (Fig. 28B) mRNA expression with increasing gestational age. *Kiss1R* mRNA was significantly higher in 11-14wpc HFA (7.9-fold, $p<0.05$) and 15-20 wpc HFA (22.3-fold, $p<0.0001$) than placenta (Fig. 28A). At 8-10wpc *Kiss1R* mRNA was 3.7-fold higher in HFA than in human placenta, although this was not significant (Fig. 29A). There was also a significant increase in *Kiss1R* expression from 8-10wpc to 15-20wpc (5.9 fold, $p<0.0001$) and 11-14wpc to 15-20wpc (2.8-fold, $p<0.001$). The 2-fold increase in *Kiss1R* expression between 8-10wpc and 11-14wpc was not significant. *SULT2A1* mRNA was significantly higher in 11-14wpc (7.3-fold, $p<0.05$) and 15-20 wpc HFA (9.4-fold, $p<0.01$) than in human adult adrenal (Fig. 29B). At 15-20wpc SULT2A1 mRNA was 2.6-fold higher in HFA than at 8-10wpc ($p<0.05$). At 8-10wpc *SULT2A1* mRNA was 3.6-fold higher in HFA than in adult adrenal, although this result was not significant (Fig. 29B). The 2.0- and 1.3-fold increase in *SULT2A1* expression from 8-10wpc to 11-14wpc and 11-14wpc to 15-20wpc, respectively, were also not significant.

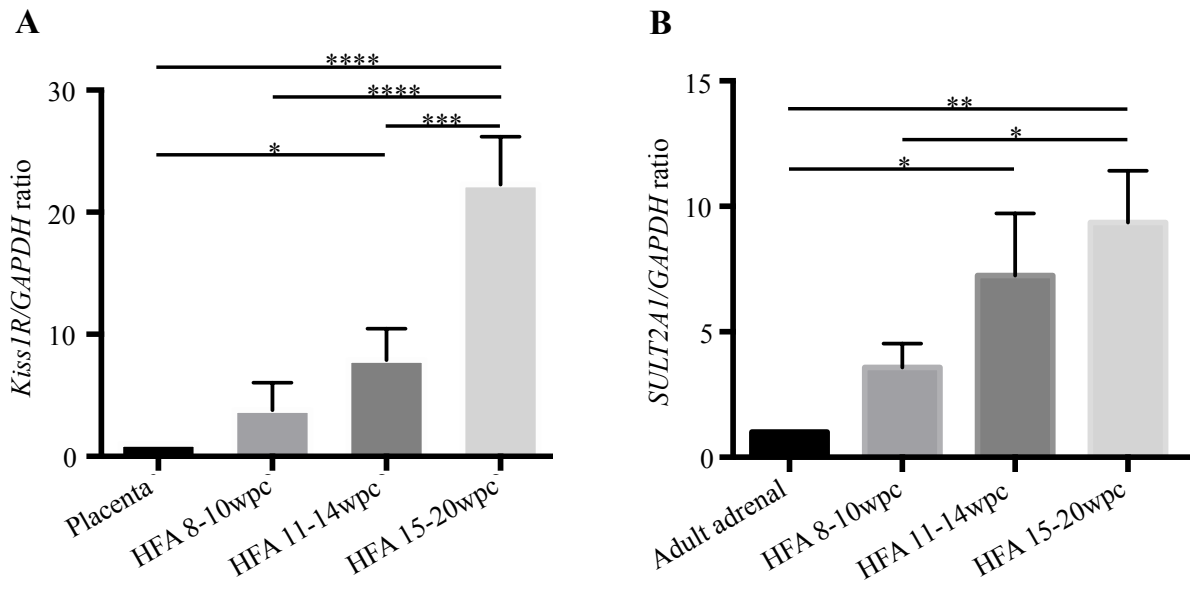


Fig. 28. A. *Kiss1R* qPCR was performed on cDNA obtained from 3 HFA samples from each stage of development (8-10wpc, 11-14wpc, 15-20wpc). Placental cDNA was used as a positive control. Data points represent the mean +/- SD from 4 independent experiments performed in triplicate. B. *SULT2A1* qPCR was performed on cDNA obtained from 3 HFA samples from each stage of development (8-10wpc, 11-14wpc, 15-20wpc). Adult adrenal cDNA was used as a positive control. Data points represent the mean +/- SD from 3 independent experiments performed in triplicate. D&E. Data are normalized to *GAPDH* expression and presented as a proportional increase or decrease from the calibrator (placenta and adult adrenal, normalized to a value of 1 for comparison). *p<0.05; **p<0.01 ***p<0.001; ****p<0.0001.

3.4 Discussion: Qualitative and quantitative expression studies of Kiss1R in the developing human adrenal

Kisspeptin and its receptor, Kiss1R, are expressed in the central nervous system, pancreas, adipose tissue, testes and spleen [119,122]. One other group has assessed the expression and localization of Kiss1R in the HFA [165]. This previous study showed robust expression of *Kiss1R*

mRNA in HFA tissue and Kiss1R protein expression in the definitive and the transitional zones (DZ and TZ) of 14-36 weeks gestation (12-34 wpc) HFA tissues by immunohistochemistry. In keeping with this study I identified expression of Kiss1R in the outer DZ/TZ. For the first time, I confirmed Kiss1R protein expression in the HFA cortex from as early as 8 wpc (10 weeks gestation) to term, 38 wpc (40 weeks gestation), by immunofluorescence. Interestingly, it was identified throughout the adrenal cortex, with expression in the inner FZ as well as the outer DZ and TZ. The reason for this discrepancy is unclear but may be accounted for by the different methodology and antibody used. However, using a second method of NR-*in situ* hybridisation, *Kiss1R* transcript was identified in the inner FZ as well as DZ/TZ.

My immunofluorescence and *in-vitro* data concord as I had hypothesised that kisspeptin stimulates DHEAS production from FZ cells. Consistent with this, SULT2A1 is localized to the FZ and converts DHEA to DHEAS. This suggests that the FZ may represent an important target for kisspeptin during pregnancy.

Quantitative evaluation shows that *Kiss1R* mRNA expression increases significantly in mid-gestation (11-20 wpc); therefore this may be a critical time point for the action of kisspeptin on the FZ. The production of DHEAS begins at ~8-10 weeks gestation (6-8wpc) but increases considerably during the second and third trimesters [20]. The increase in *Kiss1R* expression in mid-gestation (11-20 wpc) is paralleled by an increase in *SULT2A1* mRNA expression.

In conclusion, taken together, these data suggest that the FZ is an important target for kisspeptin, particularly in the second trimester.

3.4.1 Limitations and future studies

Intriguingly, my spatio-temporal studies confirm the previous report of Kiss1R expression within the outer DZ/TZ from as early as 8 wpc. Its role here is unclear and further studies are warranted.

The persistent proliferative capacity of the DZ and the observation of centripetal cellular turnover within the cortex support a centripetal model of adrenocortical cytogenesis and suggests the possible existence of stem-like cells in the outer zone of the gland [53]. It has been proposed that undifferentiated sub-capsular cells give rise to the rest of the adrenal cortex. Cells capable of repopulating the adrenocortical steroidogenic zones might be predicted to be undifferentiated and hence pluripotent. Further studies specifically examining expression of Kiss1R in the sub-capsular DZ/TZ cells would be useful to determine whether Kiss1R is expressed in differentiated steroidogenic cells, or in relatively undifferentiated steroidogenic cells (cells that express neither CYP11B1 nor CYP11B2).

It is clear from previous studies that Shh-expressing cells serve as a genuine progenitor population that contributes to the adrenal cortex homeostasis, as previously described. Previous observations indicate in rodents that the Shh-expressing sub-capsular progenitor cells and the capsular Gli-expressing cells [30,42] are integral components of the adrenocortical progenitor cell niche. SHH-expressing cells are also observed in sub-capsular clusters in the human adult adrenal [8], and this is likely to also be the case in the foetal adrenal. It could be postulated that placental kisspeptin may regulate the Hh signalling pathway and in this way regulate adrenal development. The spatio-temporal expression of SHH-expressing cells and SHH-signal-receiving cells would be interesting to characterise using serial *in situ* hybridisation with probes detecting SHH and GLI1 RNA (due to lack of availability of a good

anti-SHH antibody). The spatio-temporal expression of SHH and GLI-1-positive cells could be compared to expression of Kiss1R. These studies would be useful and important as the progenitor niche of the adrenal cortex is only just beginning to be characterized and the underlying molecular mechanisms are still unknown.

4 Results: Functional studies into the effects of kisspeptin on the human foetal adrenal cortex using *in vitro* models

4.1 Aims

The main aim of these studies is to investigate the effects of kisspeptin on foetal adrenal function, using two *in vitro* cell models comprising a human adrenocarcinoma cell line (H295R cells) and primary HFA cultures. These models will be used to interrogate the effect of kisspeptin on receptor (Kiss1R) expression and on production of DHEAS compared to other known regulators of steroidogenesis such as ACTH and CRH (measured by ELISA and LC-MS/MS). It is important to examine the possible regulation of adrenocortical steroidogenesis to obtain a better understanding of function of the foetal adrenal during pregnancy. To date only one study has examined whether kisspeptin is directly involved in steroid production in the HFA [165]. Nakamura et al previously demonstrated that kisspeptin increases aldosterone production (approximately 2-fold) in both foetal adrenocortical cells and H295R adrenal cells. In addition, Kisspeptin increased angiotensin II (Ang II)-stimulated aldosterone production by approximately 1.5-fold. Kisspeptin also increased the ability of the H295R cells to metabolize exogenously added pregnenolone to aldosterone but had no effect on the expression of aldosterone synthase (CYP11B2). The authors suggested that the high foetal/maternal levels of metastin seen during pregnancy might affect adrenal production of aldosterone. The DZ/TZ, however contributes only a small fraction of total foetal adrenal steroid production during gestation, and the DZ is capable of aldosterone secretion only near term [20]. The effect of kisspeptin on adrenal production of DHEAS, the largest steroid output from the foetal adrenal, has not previously been reported and was the main focus of these functional studies.

The H295R adrenocortical cell line was used as an *in vitro* model of the HFA to obtain preliminary data. H295R cells can differentiate towards either a zG or zF-like phenotype when treated with angiotensin II (AngII) or forskolin (Fsk) respectively [58,61]. As part of the

renin-angiotensin-aldosterone pathway, angiotensin II stimulates aldosterone production via AT1 receptors in the zona glomerulosa of the adrenal cortex [184]. It also stimulates differentiation of H295R cells towards a zG-like fate, characterized by increased production of CYP11B2 (aldosterone synthase) [58]. Activation of the HPA axis causes the release of ACTH from the anterior pituitary, which acts on its receptor, MC2R, on the surface of zF/zR cells to stimulate glucocorticoid release via intracellular cAMP production. Stimulating the cAMP pathway in H295Rs leads to their differentiation towards a zF/zR-like fate, characterised by increased production of CYP11B1 (11β-hydroxylase) [62]. Forskolin can be used to directly activate adenylate cyclase in this pathway, as H295R cells tend to be unresponsive to ACTH [185]. Initial experiments sought to reproduce those of Nakamura et al, and to ensure that H295R cells had retained their responsiveness to these compounds by examining their steroidogenic function.

Subsequently, due to limitiations of this cell line as a suitable model, as previously described, primary cultures of HFA cells were established, and both *in vitro* models were utilised in further functional studies. The direct effect of kisspeptin stimulation on Kiss1R mRNA and protein expression was investigated using real-time RT-PCR. The direct and indirect effects of kisspeptin on steroidogenesis were then examined. DHEAS was measured in the cell media by enzyme linked immunosorbent assay (ELISA) and the assay quality was checked for some experiments by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

4.2 Establishment of primary human foetal adrenal cell cultures

The importance of using primary cells, rather than cancer cell lines, for biological studies is becoming widely recognised. Kisspeptin-Kiss1R signalling plays diverse roles in human physiology, including potential roles in pathological metastatic conditions [183]. Therefore it was felt that the use of a tumour-derived cell line as a model of the HFA may limit extrapolation of any findings to an *in vivo* physiological situation. Furthermore the H295R strain demonstrates minimal response to stimulation with ACTH, which may in part be due to low expression levels of the ACTH receptor, MC2R.

Cell culture systems allow the examination of cell populations in a functional state. To simulate *in vivo* conditions as closely as possible freshly established cell strains are superior to permanent cell lines. Primary cells cannot be cultured indefinitely due to the onset of replicative senescence or aneuploidization. Hence, new cultures need to be established regularly. The procedures for primary culture is well established, however can present a challenge. At the time this work was carried out, primary HFA cell cultures were not, to my knowledge, established at any other institution in the UK. Therefore this was an invaluable resource, both within my institute and wider afield, as an *in vitro* model for studies of adrenal disorders.

4.2.1 Establishment of primary cultures

As a result of collaboration with the MRC-Wellcome HDBR in Newcastle and ICH, I established primary HFA cell cultures in our laboratory. A series of fresh HFA tissue samples from the HDBR ranging from 8 to 17 wpc (male and female sex) were obtained and primary cell cultures were established successfully on each occasion (Table 5 and Fig. 29). The medium was removed and replaced with fresh medium after 48 hours of culture. At this

time, about 70-80% of the cells had firmly attached and, thereafter, the medium was replaced every 2 days. Cells formed a 50% confluent monolayer 4-6 days after plating, and a 90% confluent monolayer 8-10 days after plating (Table 5; Fig. 30). These cultures were maintained for periods of up to 4 weeks.

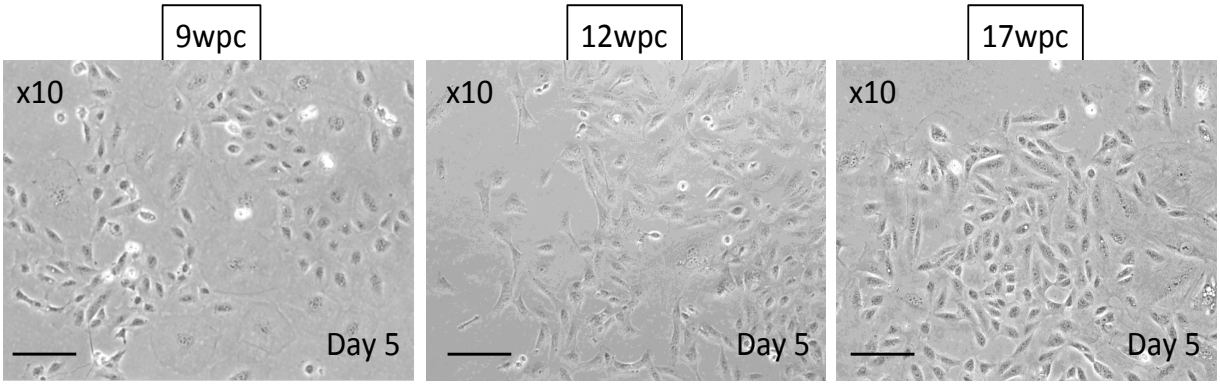


Fig. 29. Human foetal adrenal tissue from 9 wpc, 12 wpc and 17wpc in primary culture. Human foetal adrenal cells shown at low magnification (x10) growing in media containing ultroser (adherent conditions) on day 5. Scale bar: 100µm.

Table 5 Characteristics of human foetal adrenal tissue used for primary culture

Age wpc	Karyotype	50% Confluent monolayer (Day)	1st Passage (Day)	2nd Passage (Day)	3rd Passage (Day)	Complications affecting primary culture
CS23	Abnormal	10	-	-	-	Abnormal karyotype
CS23	46, XY	-	-	-	-	Infection of primary culture
8	46, XY	6	9	15	22	
8	46, XX	5	8	17	19	
9	46, XX	5	9	17	23	
9	46, XX	4	10	16	21	
9	46, XY	5	9	16	19	
10	46, XY	6	9	14	16	
11	46, XY	4	8	16	21	
12	46, XX	5	9	15	22	
13	46, XX	5	8	14	19	
15	46, XY	5	9	13	18	
16	46, XX	4	9	14	17	
17	46, XY	4	8	12	18	

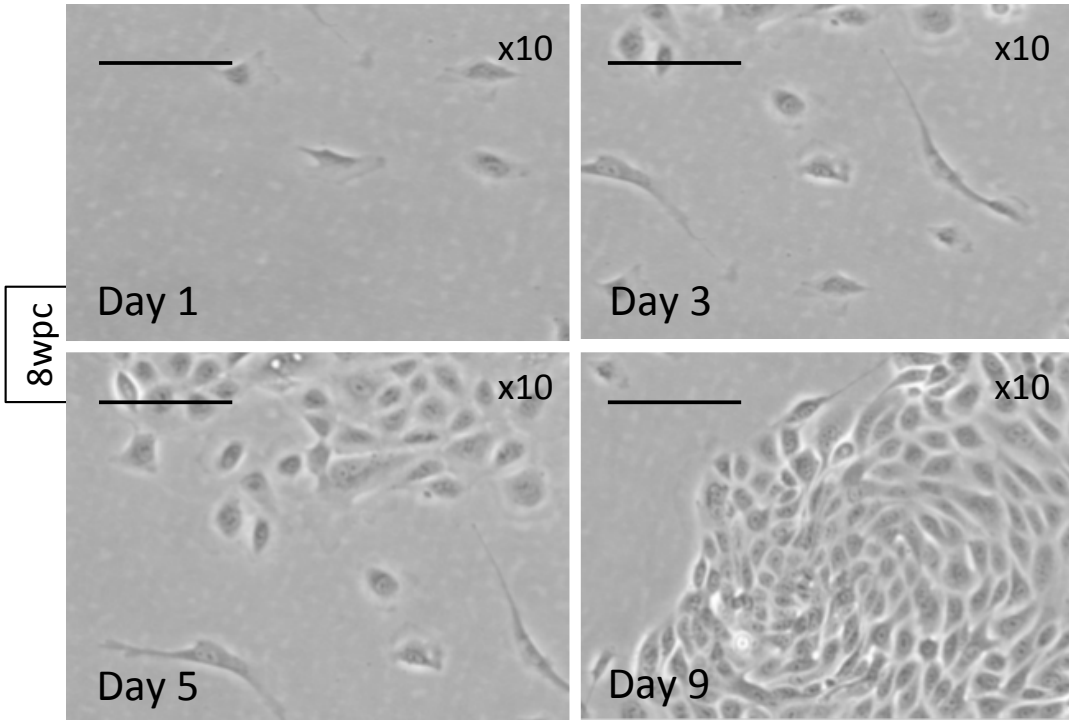


Fig. 30. The appearances of human foetal adrenocortical cells maintained in culture (cells from HFA 8wpc tissue shown growing in adherent conditions). Human foetal adrenal cells shown at low magnification (x10) growing in media containing ultroser (adherent conditions) on day 1, 3, 5 and 9 after plating. Cells were noted to have formed a 50% confluent monolayer 4-6 days after plating, and a 90% confluent monolayer 8-10 days after plating. Scale bar: 100µm.

4.2.2 Morphology and functional development of HFA cells in culture

In addition to adrenocortical cells with steroidogenic appearances (rounded nuclei and abundant lipid), a small proportion (<5%) of capsular fibroblast-like cells with elongated nuclei and multiple nucleoli were present in each primary culture of HFA cells. Fig. 31 is an example of a typical monolayer found in these cultures on day 5 and day 9 after plating. Preliminary data indicated that the mixed population of cells in culture included cells that are SF1-positive and therefore steroiodgenic (Fig. 32). In primary culture cells initially retained lipid and formed cohesive epithelial monolayers that remained stationary up until

the first passage. These cells were steroidogenic, as indicated by SF1-positivity detected by immunofluorescence (Fig. 33, HFA cells at 9wpc shown, panel A), and production of DHEAS following ACTH stimulation of cells, measured by ELISA (Fig. 33, HFA cells at 9wpc shown, panel D, E). On subsequent passages cells rapidly lost lipid, spread, and assumed fibroblastic shapes (Fig. 33, HFA cells at 9wpc shown, panel B). The morphologic changes seen appeared to be due to fibroblastic overgrowth as the number of SF1-positive cells detected by immunofluorescence was significantly less (3-fold reduction in the number of SF1-positive cells, $p<0.01$) following passage 3, compared to passage 1 (Fig. 34, HFA cells at 9wpc shown, panel C). The production of DHEAS by HFA cells was reduced 2.6-fold following passage 3 compared to passage 1, although this was not statistically significant (Fig. 33, HFA cells at 9wpc shown, panel F).

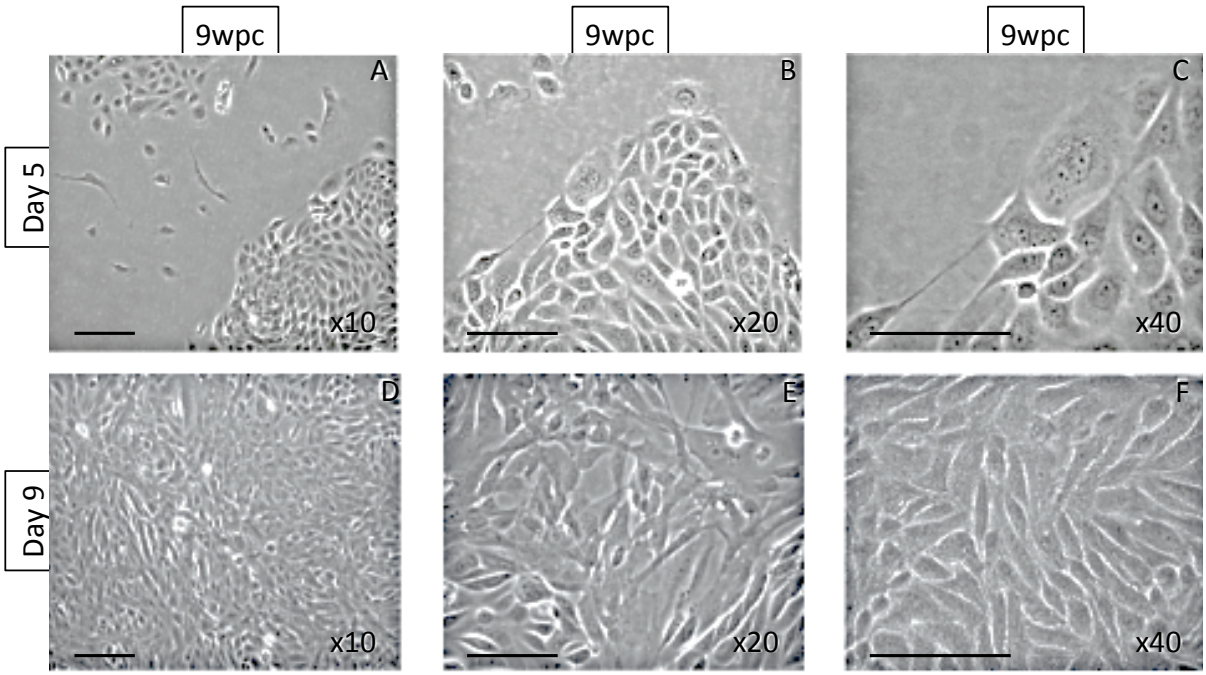


Fig. 31. Monolayer of human foetal adrenal cells from 9wpc tissue in primary culture. Human foetal adrenal tissue from 9wpc in primary culture. Human foetal adrenal cells shown growing in media containing ultroser (adherent conditions) on day 5 (A, low power x10, B, medium power x20, C, high power x40 magnification) and day 9 (D, low power x10, E, medium power x20, F, high power x40 magnification). Scale bar: 100µm.

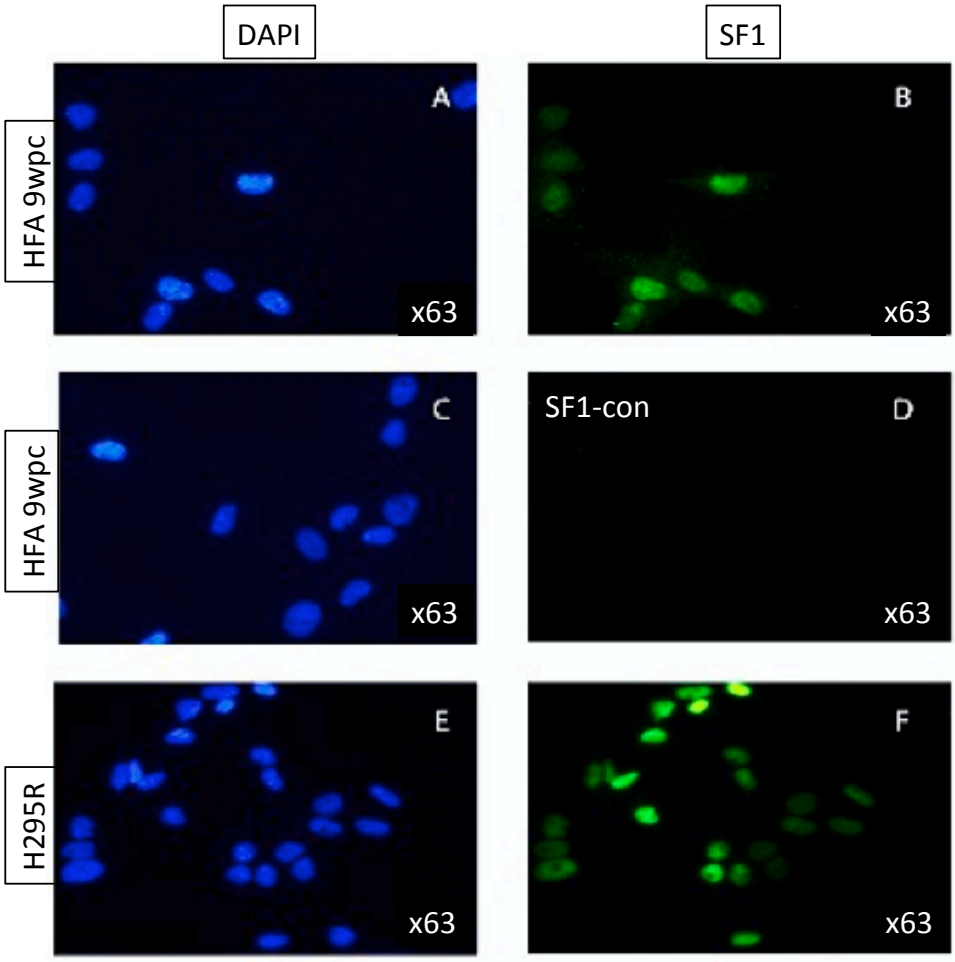


Fig. 32. Immunofluorescence studies showing that cells cultured from human foetal tissue (9 wpc) are steroidogenic factor (SF1) positive. Nuclear localisation of SF1 (green) using anti-SF1 antibody (panel A, B). No immunopositive staining is seen in the negative control (primary antibody omitted) (panel D, SF1-con). Immunopositivity for SF1 is seen in H295R adrenocortical cells (positive control) (panel F).

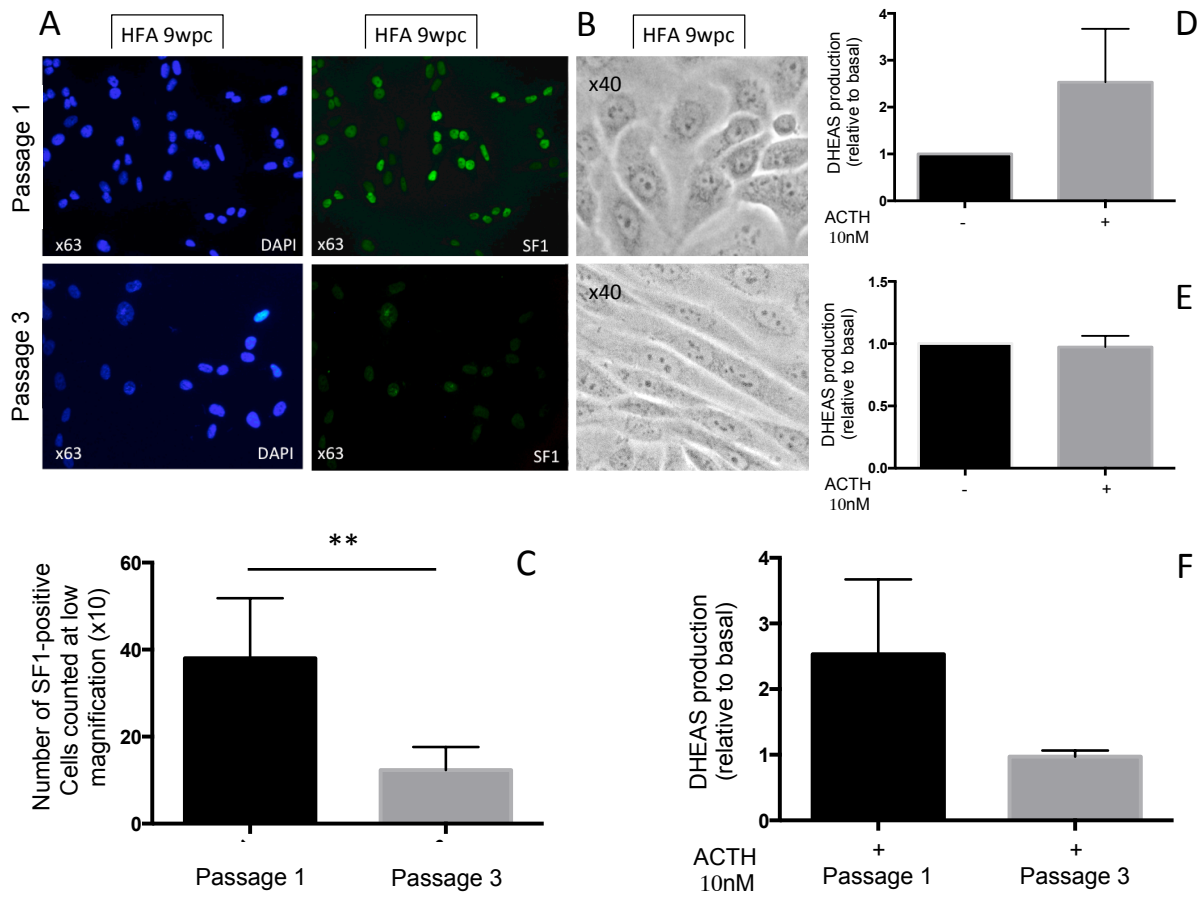


Fig. 33. Morphologic and functional changes of HFA cells in primary culture over time

In primary culture, cells retained lipid and formed cohesive epithelial monolayers that remained stationary up until the first passage (HFA at 9wpc shown). These cells were steroidogenic, as indicated by SF1-positivity detected by immunofluorescence (panel A), and production of DHEAS following ACTH stimulation, measured by ELISA (panel D, E). On subsequent passages cells assumed fibroblastic shapes (panel B). Fibroblastic overgrowth was evident as the number of SF1-positive cells detected by immunofluorescence was significantly less (3-fold reduction, p<0.01) following passage 3, compared to passage 1 (panel C). Furthermore production of DHEAS by HFA cells was reduced 2.6-fold following passage 3 compared to passage 1, although this was not statistically significant (panel F). Data points represent the mean ± SD of values from 3 independent experiments run, and are expressed as a percentage of basal level. **P<0.01.

4.3 Functional studies using in vitro models of the human foetal adrenal

Initial experiments had shown the number of SF1-positive HFA cells detected by immunofluorescence was significantly less (3-fold reduction in the number of SF1-positive cells, $p<0.01$) following passage 3, compared to passage 1 (Fig. 34, HFA cells at 9wpc shown, panel C), and the production of DHEAS by HFA cells was reduced 2.6-fold following passage 3 compared to passage 1, (Fig. 33, HFA cells at 9wpc shown, panel F). Therefore in functional studies, cells from passage 1, passage 2, and passage 3 were included for preliminary dose-response studies. All other functional studies carried out and included in the analyses that follow were performed using passage 1 cells only.

4.3.1 Preliminary time course and dose-response studies

Time course experiments examining the effect of kisspeptin on DHEAS production in H295R cells were carried out. HFA cells were unavailable for use in these studies. A concentration of 100nM kisspeptin was selected for time course experiments following the studies using H295R cells carried out by Nakamura et al [165]. At 24 hours, stimulation of cells resulted in a significant increase in DHEAS production (Fig. 34. Treatment of cells with kisspeptin resulted in an over 5-fold increase in DHEAS production measured by ELISA, $p<0.05$).

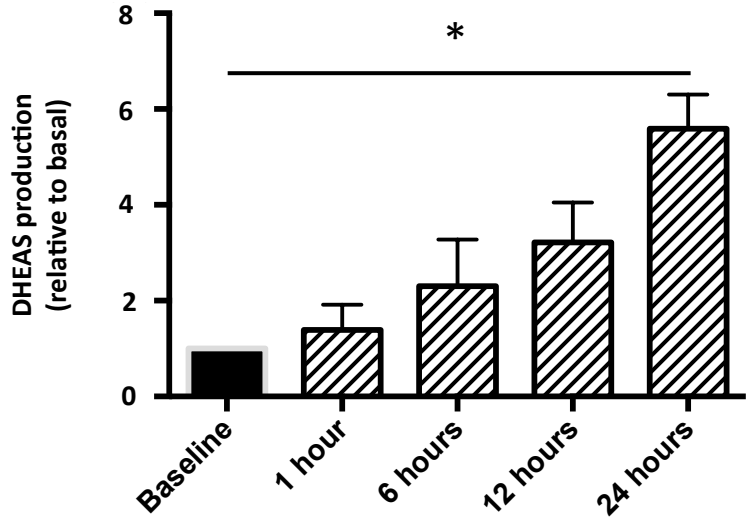


Fig. 34. Time-dependent effects of kisspeptin on DHEAS production in H295R cells.

Cells were incubated with kisspeptin 100nM or serum-free media alone (basal control, untreated cells), (-) no treatment; (+) treatment added. Steroid level in the media was determined by enzyme immunoassay and corrected for protein content. Data points represent the mean ± SD of values from 3 independent experiments run, and are expressed as the fold over basal level (normalised to a value of 1). **P<0.01.

The kisspeptin concentration used in experiments was supraphysiological (100x maternal circulating concentrations). This was decided on the basis of dose response studies, which showed a significant increase in DHEAS compared to untreated H295R cells with 10nM ($p<0.01$) and 100nM ($p<0.05$). Only 100nM kisspeptin produced a significant increase in DHEAS compared to no treatment in 8-10wpc ($p<0.05$) and 15-20 HFA ($p<0.0001$) cells (Fig. 35 A B and C, respectively). Using LC-MS/MS (Fig. 35 D-E) to measure DHEAS produced a similar finding with 100nM kisspeptin resulting in a significant increase ($p<0.05$) compared to no treatment in H295R cells (D) and HFA 8-10wpc cells (E). LC-MS/MS is the gold standard method for quantifying steroid production; however mass spectrometry results were unavailable for all the time points of interest.

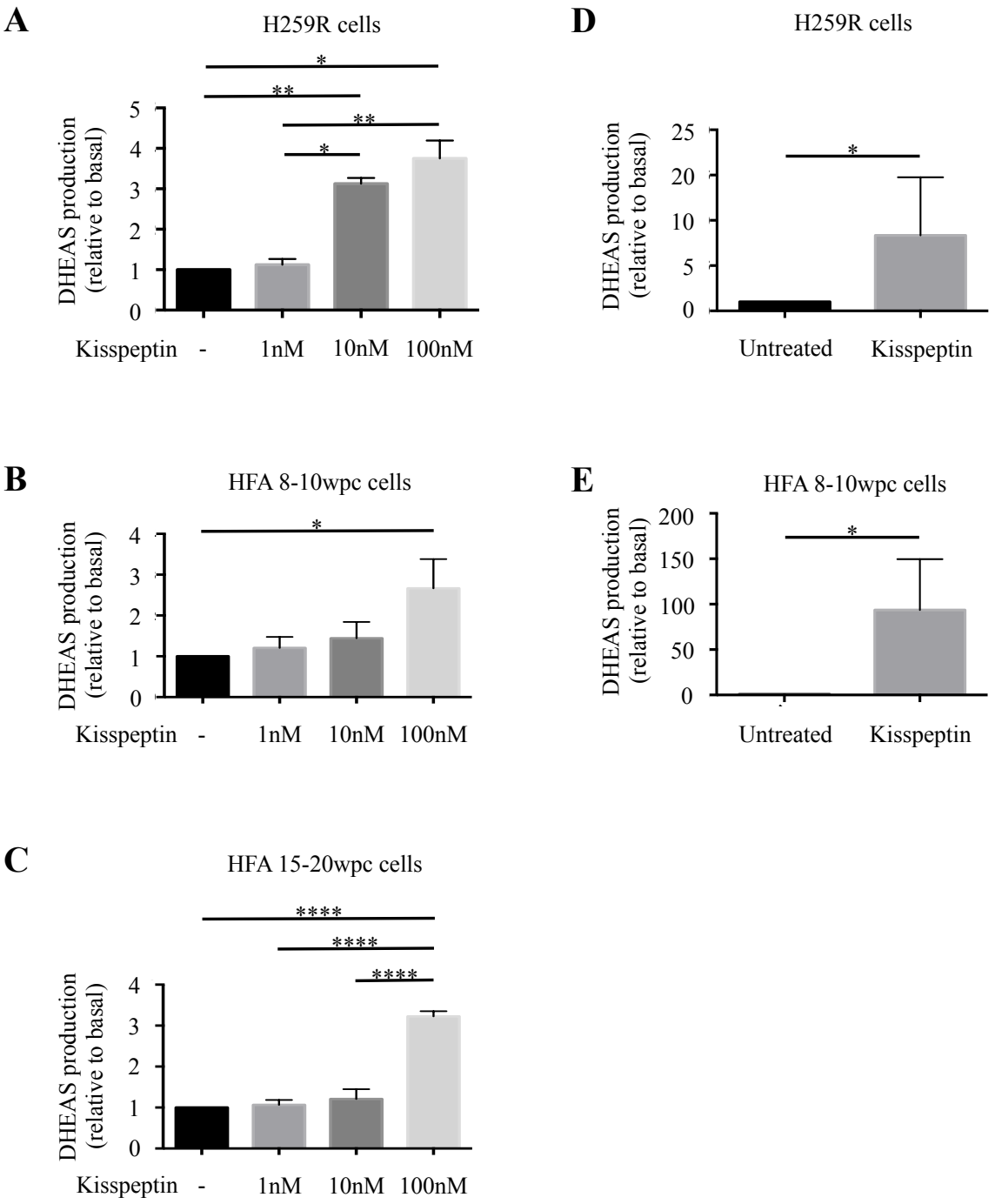


Fig. 35. Kisspeptin dose response studies.

A-C. DHEAS production (ELISA) by H295R and HFA cells following kisspeptin treatment. H295R cells (A), 8-10wpc HFA cells (passage 1, 2 and 3) (B) and 15-20wpc HFA (passage 1 and 2) cells (C) were incubated with kisspeptin 1nM, 10nM, 100nM, or no treatment (-) for 24 hours. Data points are mean +/- SD from 3 independent experiments run in triplicate

and expressed as the fold over basal level (normalised to a value of 1). (-), no treatment; (+) treatment added.

D-E. DHEAS production (LC-MS/MS) by H295R (D) and 8-10wpc HFA cells (passage 1) (E) following 24 hours 100nM kisspeptin treatment. Data points are mean +/- SD derived from 3 independent experiments run in triplicate and expressed as the fold over basal level (normalised to a value of 1). *p<0.05; **p<0.01; ****p<0.0001.

4.3.2 The effect of kisspeptin treatment on Kiss1R mRNA and protein expression in H295R and HFA cells (passage 1)

Treatment of H295R adrenocortical cells with kisspeptin resulted in a significant (60%) decrease in *Kiss1R* mRNA expression (Fig. 36A; $p<0.05$). A significant decrease (34%) in *Kiss1R* mRNA expression was also observed in 15-20 wpc HFA cells (passage 1) (Fig. 36C; $p<0.05$) but not 8-10 wpc HFA cells (passage 1) (16% decrease, Fig. 36B) in response to kisspeptin treatment. Kisspeptin treatment also resulted in a significant decrease in Kiss1R protein levels in H295R adrenocortical cells (53.5% decrease from baseline; $p<0.05$) (Fig. 36D). Kiss1R protein expression was decreased in 8-10 week HFA cells (passage 1) (8.3% reduction; Fig. 36E) and 15-20 week HFA cells (passage 1) (8.9% reduction; Fig. 37F) in response to kisspeptin treatment, however these differences were not significant.

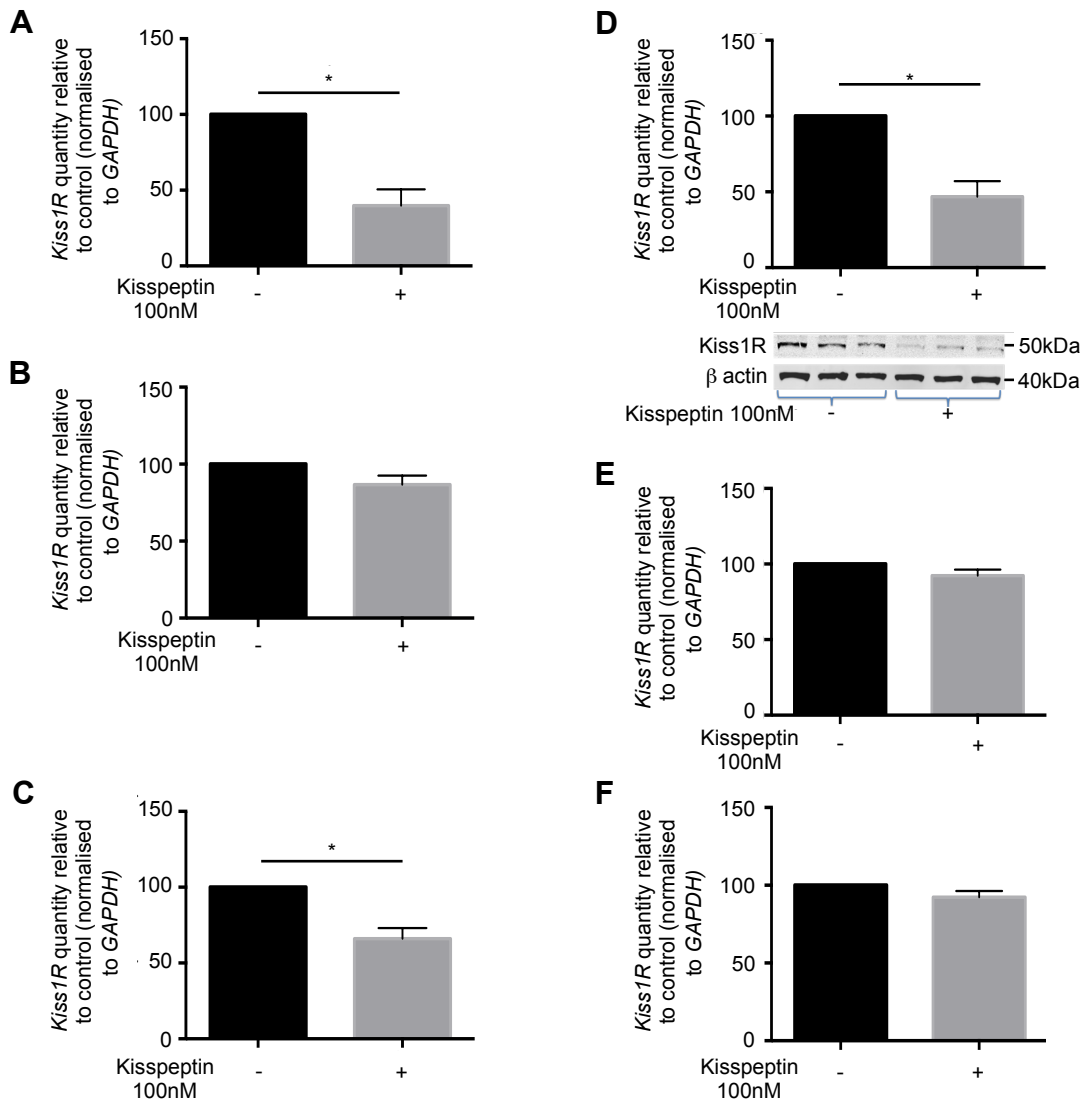


Fig. 36. The effect of kisspeptin on Kiss1R expression in H295R and primary HFA cells (passage 1).

A-F. The effect of kisspeptin treatment on *Kiss1R* mRNA and protein expression. Treatment with 100nM kisspeptin for 24 hrs significantly reduced *Kiss1R* mRNA expression in H295R cells (A). This was also evident in HFA 15-20wpc cells (passage 1) (C) but not 8-10wpc cells (passage 1) (B). Data are normalized to *GAPDH* expression and presented as a proportional increase or decrease from the control (unstimulated cells, normalized to a value of 100 for comparison). Data points represent the mean +/- SD from 3 independent experiments performed in triplicate. D-F. Densitrometric analysis of Western blots

showed a significant reduction in Kiss1R protein following treatment of H295R cells with 100nM kisspeptin for 24 hours (D) but not 8-10wpc HFA cells (passage 1) (E) or 15-20wpc cells (passage 1) (F). Data points represent the mean +/- SD from 3 independent experiments performed in triplicate. β-actin was used as a loading control. *p<0.05.

4.3.3 The effect of kisspeptin and known adrenal regulators on DHEAS production (measured by ELISA and LC-MS/MS) in H295R and HFA cells (passage 1).

Kisspeptin significantly increased DHEAS secretion from H295R, 8-10wpc and 15-20wpc HFA cells 3.7-fold ($p<0.05$, Fig. 37A), 2.7-fold ($p<0.05$; Fig. 37B) and 4.0-fold ($p<0.05$; Fig 37C) compared to untreated cells, respectively. DHEAS production from 8-10wpc and 15-20wpc HFA cells following kisspeptin treatment was similar to that produced by ACTH (3.5-fold ($p<0.01$) and 4.1-fold ($p<0.05$) compared to untreated cells, respectively). Kisspeptin with forskolin increased DHEAS secretion 8.6-fold from H295R cells compared to untreated cells ($p<0.0001$) (Fig. 37A), which was 2.3-fold ($p<0.01$, Fig. 37A) and 1.4 fold ($p<0.05$, Fig. 37A) higher than kisspeptin or forskolin alone, respectively. Kisspeptin with ACTH also increased DHEAS production 4.5-fold ($p<0.001$; Fig. 37B) and 9.1-fold ($p<0.0001$, Fig. 38C) from 8-10wpc and 15-20wpc HFA cells compared to untreated cells, respectively. This was 1.7-fold ($p<0.05$; Fig. 37B) and 2.3-fold ($p<0.01$; Fig. 37C) higher than with kisspeptin alone in 8-10wpc HFA cells and 15-20wpc HFA cells, respectively and 2.2-fold ($p<0.01$; Fig. 37C) higher than with ACTH alone in 15-20wpc HFA cells. Consistent with these data, when the cell media was analysed by LC-MS/MS, there was a significant increase in DHEAS following kisspeptin treatment from H295R (10-fold) and 8-10wpc HFA cells (>50-fold) (Fig. 35D, E). LC-MS/MS measurement of steroids also clearly demonstrates shunting of metabolites down the androgen pathway, with preferential production of DHEAS compared to other

steroids in response to stimulation of H295R and HFA (8-10wpc) cells by kisspeptin (Fig. 38). My ELISA data confirm that kisspeptin can significantly increase DHEAS production from H295R and 8-10 / 15-20wpc (10-22 weeks gestation) HFA cells. The increase of DHEAS described with the LC-MS/MS assay is much higher than with the one described with the immunoassay. The greater increase for the HFA experiment when calculated as fold change can be ascribed to the baseline values being much lower. The values before protein correction for the DHEAS concentrations are compared in Table 6 below. HFA medium must contain significant amounts of steroids that are read in the ELISA as DHEAS, whereas this is not the case for H295R cells. Another possibility is that DHEAS might have been desulfated however no evidence was found for this in practice and pre-analytical steps verified the specificity of the LC-MS/MS assay for DHEAS.

DHEAS production (measured by ELISA) by H295R, 8-10wpc and 15-20wpc HFA cells following CRH treatment was similar to that produced by ACTH and kisspeptin treatment alone (4.0-fold ($p<0.01$), 4.0-fold ($p<0.001$) and 2.5-fold ($p<0.05$) compared to untreated cells, respectively) (Fig. 37D-F). Compared to kisspeptin and CRH alone, treatment of H295R cells with a combination of kisspeptin and CRH resulted in a significant decrease (2.6-fold; $p<0.01$ and 2.8-fold; $p<0.01$) in DHEAS production, respectively (Fig. 37D). The same pattern was observed in 8-10wpc HFA cells (3.1-fold ($p<0.01$) and 4.7-fold ($p<0.001$) decrease of DHEAS (measured by ELSIA) compared to kisspeptin and CRH treatment alone, respectively) (Fig 37E) and 15-20wpc HFA cells (3.5-fold ($p<0.001$) and 2.2-fold ($p<0.05$) decrease of DHEAS compared to kisspeptin and CRH treatment alone, respectively) (Fig 37F).

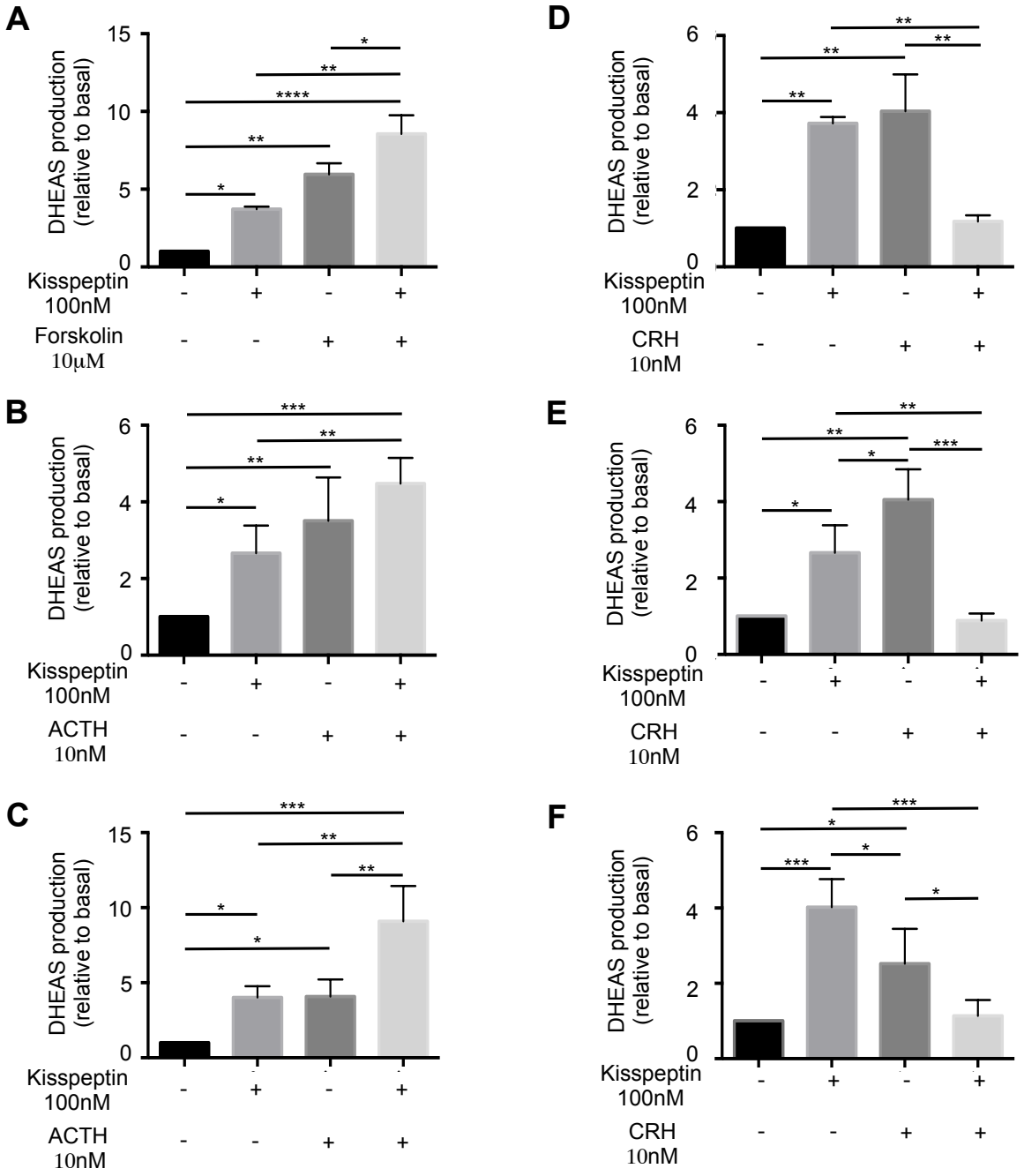


Fig. 37. The effect of kisspeptin, ACTH and CRH treatments on DHEAS production (measured by ELISA) by H295R and primary HFA cells (passage 1).

A-C. DHEAS production (ELISA) by H295R and HFA cells following kisspeptin and CRH treatments. H295R cells were incubated for 24 hours with 100nM kisspeptin and 10mM forskolin individually or together (A). 8-10wpc HFA cells (B) and 15-20 wpc HFA cells (C) were incubated for 24 hours with 100nM kisspeptin and 10nM ACTH individually or together. D-F. DHEAS production (ELISA) by H295R and HFA cells following kisspeptin and

CRH treatments. H295R cells (D), 8-10wpc HFA cells (E) and HFA cells 15-20wpc (F) were incubated for 24 hours with 100nM kisspeptin or 10nM CRH individually or together. Data points are mean +/- SD from 3 independent experiments run in triplicate and expressed as the fold over basal level (normalized to a value of 1). (-), no treatment; (+), treatment added. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

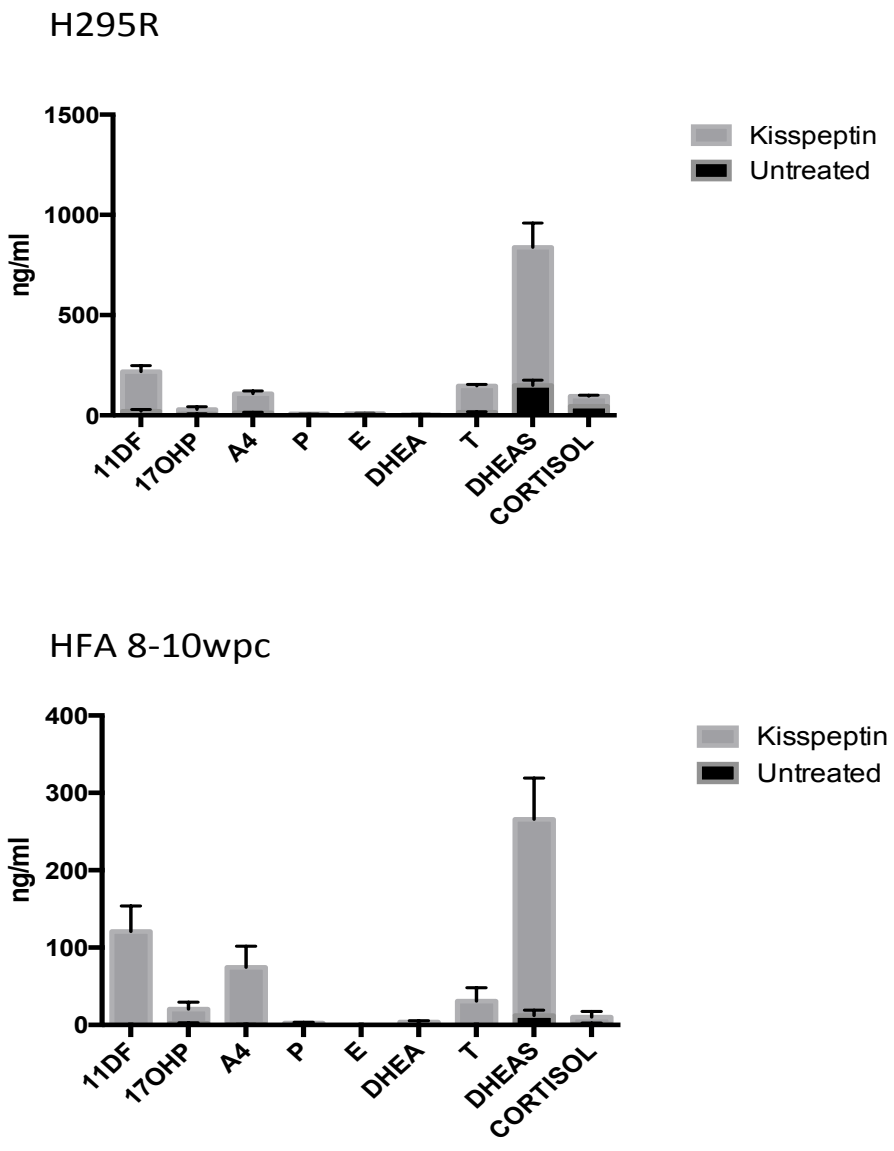


Fig. 38. The effect of kisspeptin on steroidogenesis (measured by LC-MS/MS) by H295R and primary HFA cells (8-10wpc; passage 1 cells).

Steroid production (LC-MS/MS) by H295R and 8-10wpc HFA cells following 24 hours 100nM kisspeptin treatment. Data points are mean +/- SD derived from 3 independent experiments run in triplicate (11DF, 11-deoxycortisol, 17OHP, 17-hydroxyprogesterone, A4, androstenedione, P, progesterone, E, oestrogen, DHEA, dehydroepiandrosterone, T, testosterone, DHEAS, dehydroepiandrosterone sulphate).

Table 6. ELISA vs LCMS/MS data

H295R cell line

	DHEAS concentration ng/ml	DHEAS concentration ng/ml	DHEAS concentration ng/ml	Mean DHEAS concentration ng/ml	Mean fold change (relative to untreated which =1)
Untreated (ELISA)	105.9	141.8	110.4	119.4	1
Kisspeptin 100nM (ELISA)	393.16	551.5	392.85	445.8	3.7
Untreated (LCMS)	129.8	168	12.5	103.4	1
Kisspeptin 100nM (LCMS)	603.6	775.3	196.9	525.3	8.32

HFA 8-10 week (passage 1,2, and 3)

	DHEAS concentration ng/ml 8wpc	DHEAS concentration ng/ml 9wpc	DHEAS concentration ng/ml 10wpc	Mean DHEAS concentration ng/ml 8-10wpc	Mean fold change (relative to untreated which =1)
Untreated (ELISA)	138.9	85.6	100	108.2	1
Kisspeptin 100nM (ELISA)	255.8	270.1	301.2	275.7	2.7
Untreated (LCMS)	2.92	4.8	2.32	3.3	1
Kisspeptin 100nM (LCMS)	86.5	552.8	312.5	317.2	93.5

4.4 Discussion: Functional studies of kisspeptin stimulation in *in vitro* models of the human foetal adrenal

KISS1R is a G-protein coupled receptor (GPCR). Kisspeptin-KISS1R signalling is best characterized in GnRH neurons and in these cells, KISS1R undergoes both kisspeptin-triggered and kisspeptin-independent signalling, internalization and recycling [186]. This ensures a dynamic population of functional cell-surface receptors and tight regulation of the biochemical response. In HFA cells, kisspeptin treatment resulted in a significant decrease in *Kiss1R* mRNA expression in adrenocortical tumor (H295R) and second trimester HFA cells.

This was paralleled by a significant decrease in Kiss1R protein levels in H295R cells. This data is novel and we hypothesise that high circulating kisspeptin levels may down-regulate *Kiss1R* expression in the HFA to regulate signaling and therefore ensure tight control of steroidogenesis throughout pregnancy. This process of desensitisation is a recognized phenomenon of many other GPCRs [187] as well as kisspeptin [188].

The kisspeptin effect on DHEAS production measured using immunoassay in all of the studies perfomed. In a number of experiments the cell media was also analysed using LC-MS/MS. Quantification of hormonal steroids and their precursors using immunoassay can be problematic, particularly for low concentration analytes due to assay interference by other endogenous steroids. Mass spectrometry offers improved specificity over immunoassay and can be highly sensitive. Gas chromatography mass spectrometry (GC-MS) with use of stable isotopically labelled internal standards is considered the gold standard method for serum steroid analysis [189]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) compares well to GC-MS in terms of accuracy, precision and sensitivity but allows simplified sample preparation [189]. Multi-targeted steroid analysis (steroid panelling) is adaptable for use in a number of matrices including cell media. LC-MS/MS analyses of my *in vitro* experiments clearly show the preferential production of DHEAS over other steroid metabolites in response to kisspeptin stimulation of H295R and 8-10wpc HFA cells.

Kisspeptin-stimulated production of DHEAS by primary HFA cells and H295R cells was comparable to stimulation with ACTH or CRH alone. Furthermore, kisspeptin in combination with ACTH appears to augment this effect. In contrast, CRH in combination with kisspeptin significantly decreased DHEAS production (see also later, Chapter 5.5 Discussion: Clinical study). The independent agonist effects of kisspeptin and CRH on DHEAS production

compared to their apparent antagonistic effect in combination is an interesting observation and warrents further study. The combination of CRH and kisspeptin may result receptor desensitization, which represents an important physiological process that prevents GPCRs, such as Kiss1R, from overstimulation due to prolonged agonist exposure by signal attenuation or termination [190,191]. The classical model of GPCR desensitization involves three processes: (1) receptor phosphorylation and subsequent uncoupling of the receptor from its cognate G protein, (2) receptor sequestration (internalization) to intracellular compartments, and (3) downregulation. Additional possible mechanisms for the observed reduction in DHEAS production when kisspeptin and CRH were administered together to H295R and HFA cells, may be found by examining other reported *in vivo* hormone interactions:

1) Direct, steroid-independent down-regulation of CRH mRNA expression by ACTH has been demonstrated in central neurons, in both therapeutic and physiological contexts [192]. Modulation of CRH synthesis may prove to be one of the mechanisms by which ACTH exerts its profound and diverse effects on CNS function. *In vivo* placental synthesis of CRH may be modulated in a similar manner by kisspeptin or *vice versa*.

2) Luteinizing hormone (LH), in synergy with follicle stimulating hormone (FSH), stimulates normal follicular growth and ovulation. FSH is frequently used in assisted reproductive technology (ART), however recent studies have shown that maturing follicles become increasingly sensitive to, and ultimately dependent on, the presence of LH for their development [70]. The ovary comprises of two cellular components, which are stimulated independently by LH and FSH, leading to the production of ovarian steroids [193]. Ovarian steroidogenesis in the preovulatory follicle takes place through LH receptors on theca and FSH (possibly plus LH) receptors on granulosa cells [194]. Maintaining homeostasis often requires conditions to be limited to a narrow range. The ovary has a minimum requirement

level (threshold requirement) for FSH below which follicular development does not occur [195]. Furthermore, it has been shown that FSH threshold is not fixed for any given follicle, but depends on the developmental stage and varies over time [196]. The concept of the LH therapeutic window may interplay with this and is an area of on-going studies. Low levels of LH impair follicular growth and oocyte maturation, and hence androgen and oestrogen synthesis. High levels result in follicular atresia and compromise of oocyte development, and suppression of granulosa cell proliferation. In ART one protocol for a poor responder utilizes a GnRH analogue at the beginning of the cycle, resulting in a flare effect of FSH and LH and augmenting the folliculogenesis already in progress [70]. Though follicles are recruited with this method, the excess of LH in the early part may be deleterious to the growing follicle. The experiments conducted in this work to examine the effect of kisspeptin and CRH, alone and in combination, on DHEAS production, were based on preliminary dose-response and time course studies. Earlier or later time points, or different dose ratios of the two hormones in combination may have resulted in increased or decreased DHEAS production and this would be interesting to explore in future work.

Thus kisspeptin may work in concert with CRH and ACTH to regulate HFA function and therefore the balance of oestrogens during pregnancy. The placenta is the primary source of oestrogen and the concentration of oestrogen increases with progressing gestational age. The timing of these interactions may be critical as ACTH levels remain fairly steady throughout pregnancy and circulating kisspeptin levels rise steadily between the first and third trimesters. Consequently as pregnancy progresses, kisspeptin may work in tandem with ACTH to enhance DHEAS, the production of oestrogens and the maintenance of pregnancy. In support of this hypothesis, low levels of kisspeptin, particularly in early pregnancy, are associated with greater miscarriage risk [163].

In conclusion these data support the hypothesis that kisspeptin-Kiss1R signalling may be a key regulator of HFA steroidogenesis.

4.4.1 Limitations and future studies

Within the time-scale and remit of this study I was unable to explore the functional role of Kiss1R expression seen in the outer DZ/TZ of the HFA. Nakamura et al previously reported an increase in aldosterone production *in vitro* from H295R cells in response to stimulation with kisspeptin [165]. *In vivo* DZ steroidogenesis occurs only late in the 3rd trimester as previously described. This suggests kisspeptin-Kiss1R signalling in the outer cortex may be important for other roles, such as growth and differentiation, early in adrenal development. This hypothesis is supported by the observation that in the postpartum period the foetal adrenal undergoes significant remodelling with involution of the large FZ, suggesting that placental factors, which are pregnancy specific, are likely to act to maintain foetal adrenal growth and differentiation *in utero*.

Proliferation of isolated DZ and FZ cells could be assessed by an MTS assay where absorbance readings are proportional to cellular metabolic activity. Methods of directly labelling DNA in proliferating cells have been extensively reported including BrdU (5'-bromo-2'-deoxyuridine), that is incorporated into the proliferating DNA during S-phase of cell division [197]. The limitation is that its detection is lengthy and requires harsh treatment of tissue sections to give access of anti-BrdU antibody to nucleosides in genomic DNA. A quicker, simpler, and highly sensitive method relying on incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into de novo DNA was subsequently developed [198]. EdU is a viable

alternative for direct DNA labelling of proliferating cells and could be used to quantify the effect of kisspeptin stimulation on proliferation of HFA cells, when compared to untreated. Cells could be double labelled with Kiss1R antibody following EdU chemistry to determine if proliferating cells are those that also express Kiss1R.

To investigate the effect of kisspeptin on differentiation, the expression of zone-specific markers could be analysed in DZ and FZ cells by qPCR. Markers quantified might include: CYP11B2 (DZ), CD56 (DZ), CYP11B1 (TZ), and SULT2A1 (FZ) at baseline and following kisspeptin stimulation. An increase in expression of SULT2A1, for example, in DZ cells following stimulation would indicate differentiation towards a FZ phenotype.

All my in vitro functional work using primary HFA cultures were limited by the age of material available. Primary culture of late trimester HFA tissue is not possible due to ethical and legal restrictions. *In vitro* studies of the role of kisspeptin and its interaction with other pregnancy-specific hormones in late pregnancy are difficult to conduct due to lack of appropriate cell models. Furthermore, *in vitro* studies using cell lines and primary cell culture may be limited in extrapolation to *in vivo*. Studies involving animal models are complex and time-consuming and may also be limited in extrapolation to *in vivo* due to species-specific differences in adrenal development.

The adrenal cortex is a very dynamic organ, in which steroid secretion correlate with morphology and structure according to external stimuli or surrounding environmental conditions. All pathways implicated in steroidogenesis and adrenal growth are closely interconnected and probably dependent on the extracellular matrix and the cytoskeleton. It has been shown that the cell environment is important to dictate the nature of steroids secreted, and even the activation of transcription factors [199–202]. For example, in isolated

cells in culture, ACTH inhibits cell proliferation to favor steroid secretion [203]. It is now relatively accepted that ACTH is preferentially a differentiation factor controlling steroid secretion rather than a proliferation factor. However, ACTH favours cell survival when viability is compromised, a protective effect occurring only when the adrenal glands are intact. ACTH loses its protective effects when the adrenal architecture is disrupted [204]. Quartering of the glands enhances basal apoptosis and, interestingly, abolishes ACTH-induced inhibition of apoptotic DNA fragmentation, without altering ACTH-induced corticosterone secretion. These data suggest that the global organ architecture is required for modulation of adrenal cell survival by ACTH [204]. The precise mechanisms of interactions between the ECM and integrin receptors with the cytoskeleton and intracellular kinases is beginning to emerge but is yet to be correlated with *in vivo* physiology. The results from primary cell experiments detailed in my work followed from initially disrupting adrenocortical structure and separating the cells into a monolayer. The fact that significant results were obtained in a monolayer may suggest that the effects in the intact organ may be even greater than shown here.

Emerging innovative *in vitro* techniques such as organoid culture and engineered ECM are promising approaches to studying such a small yet complex organ like the foetal adrenal gland. Organoid culture has emerged in recent years as a valuable tool to study several aspects of stem cell biology, tissue morphogenesis, and lineage specification [205,206]. Organoids are self-organizing three-dimensional structures that are derived from stem cells and exhibit organotypic anatomic and functional features. Organoids can be grown *in vitro* from pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells) or organ-specific stem cells [206]. Organoid cultures have been established for different mouse and human tissues, and have been proven to be valuable models for studying different

aspects developmental biology such as tissue morphogenesis, organogenesis, differentiation, heterotypic interactions between different cell types, and the effects of the ECM on cell differentiation and behavior. However, a current limitation of organoid cultures is the organoid dependence on a suitable three-dimensional matrix. Engineered synthetic ECM has emerged as an attractive approach to mimic the original three-dimensional microenvironment of a given organ or tissue [207].

5 Results: Proof of concept clinical study

5.1 Aim

Circulating kisspeptin levels increase dramatically during pregnancy [158] and have an important role in placentation by regulating placental invasion into the maternal uterine wall [118,122,130]. Circulating kisspeptin levels are reduced in women with intrauterine growth retardation and preeclampsia [155,156] and low maternal levels in early pregnancy have been associated with greater miscarriage risk [163]. Therefore, kisspeptin may be a novel endocrine marker of functional placental tissue and low placental kisspeptin may be associated with serious obstetric complications. The role of kisspeptin in pregnancy and the mechanisms underlying these associations are unclear. A prospective observational study of patients with singleton, uncomplicated pregnancies was undertaken. The aim of this was to ascertain whether kisspeptin-Kiss1R signalling might be a regulator of HFA function *in vivo.*

5.2 Participant demographics, progress and outcomes

33 pregnant women of mean age 26.8 ± 6.1 yrs were recruited. The median gravidity and parity of the women were 1 (range 1-4) and 0 (range 0-2), respectively. The participant demographics are detailed in Table 7 and 8. Two subjects (21 and 23) moved area before completion of the study (after visits 2 and 3, respectively). Two babies were born prematurely at 26 and 33 weeks gestation (18 and 30, respectively) with normal birth weights of 950 and 1340g (BW SDS 0.84 and -1.95), respectively (Table 9). Three participants (6, 9 and 22) developed pre-eclampsia. Maternal serum biomarkers of placental function were not significantly different at visits 1 and 2 between those women who had normal pregnancies and those who later developed complications including PE (Table 10). Interestingly, participant 6 was noted to have low PlGF 50.61 pg/ml at visit 4, and participant 22 was noted to have low PlGF 76.17 pg/ml at visit 3. Both these women

developed PE in the 3rd trimester, and their infants, born at 39.3 and 37.1 weeks gestation respectively were small for gestational age, BW 2420 and 1720g (BW SDS -2.42 and -3.30); these two infants were both delivered following induction of labour for pre-eclampsia and intra-uterine growth retardation (Table 9). The remaining subjects had term deliveries, mean gestation 39.96 ± 1.18 (range 37.14 - 42.14) and BW SDS -0.71 ± 0.87 (-3.30 - 0.57).

Table 7. Details of the participants, the timing of the antenatal assessments and pregnancy outcome

Characteristic	Mean ± SD	Range	N
Age (years)	26.8 ± 6.1	17.0 - 37.0	33
BMI	25.4 ± 5.3	18.8 - 41.1	33
Gestational age (weeks)			
Visit 1	20.35 ± 0.68	19.29 - 22.57	31
Visit 2	28.07 ± 0.75	26.29 - 29.86	32
Visit 3	34.42 ± 0.75	33.00 - 36.14	28
Visit 4	38.09 ± 0.62	36.00 - 39.71	22
Gestation at delivery (weeks)	39.33 ± 2.83	26.86 - 42.14	31
Birth weight (grams)	3006 ± 650	950 - 3680	31
Birth weight SDS	-0.698 ± 0.913	-3.30 - 0.840	31

N= total number of subjects assessed

5.3 Foetal adrenal volume and kisspeptin levels in pregnancy

Foetal adrenal volumes (FAV) increase steadily during pregnancy (Fig. 39A & Table 8). Median FAVs were 0.19 cm^3 (IQR 0.08-0.48; n=31), 0.52 cm^3 (0.26-1.53; n=32), 1.52 cm^3 (0.94-2.40; n=28) and 2.16 cm^3 (1.17-7.87; n=23) at antenatal visits 1-4, respectively. The range of FAVs increase as gestation advances but there are significant increases in FAV between visits 1 and 2 ($p<0.01$), visits 1 and 3 ($p<0.001$), visits 1 and 4 ($p<0.001$) and visits 2 and 4 ($p<0.01$). The difference in FAV between sequential visits is consistent with an overall increase during pregnancy. Between antenatal visit 1 and 2 the median increase in FAV is 0.29 cm^3 (IQR 0.13-1.14; n=31), 0.61 cm^3 (IQR -0.06-1.59; n=29) between antenatal visits 2 and 3, and 0.41 cm^3 (IQR -0.13-2.64; n=21) between antenatal visits 3 and 4. The differences in FAV between sequential visits were not statistically significant (Fig 40A).

Postnatally rapid involution of the adrenal is seen. Median FAVs were 0.105 cm^3 (IQR 0.0775-0.3175; n=8), 0.12 cm^3 (0.07-0.14; n=9), 0.04 cm^3 (0.037-0.05; n=14) at postnatal visits 1-3, respectively (Fig. 41). There is a significant decrease postnatally in FAV between visits 1 (days 1-2 of life) and visit 3 (6-8 weeks of life) ($p<0.01$). In keeping with previous US studies of the neonatal adrenal [208], postnatal involution of the adrenal was seen in our cohort of neonates and occurred following delivery, suggesting parturition itself is the basis for foetal adrenal involution. The adrenals were seen to decrease in ultrasonographic volume from d 1 to 7 of life, however this was not significant. A significant decrease in adrenal volume was seen between the first day of life and ~2 months of life, regardless of gestational ages and sex. Thus, parturition itself would appear to be the basis for foetal adrenal involution, further supporting a key role for placental factors in maintaining the

foetal adrenal and generating adrenal androgens. It has been previously reported that on day 4 of life the adrenal looses 25% of its mass; at 1 month of age, it has lost 50%, and at 1 year it has lost 75% of its birth mass [98]. The present findings disagree with previous reports that involution of the adrenal is related to gestational age rather than birth [24]. The study by Midgley et al used urinary levels of 3β-OH-5ene steroids as a measure of urinary foetal zone steroids and suggested that the foetal adrenals maintain high androgen levels for 3–4 wk after term. In this study, in parallel to postnatal US measurements of the adrenal, urine had been collected from neonates and stored for analysis of steroid metabolites by LC-MS/MS with the aim of documenting functional change over time. Unfortunately due to time and resource pressures on a clinical service these samples are yet to be processed but would form part of ongoing collaborative studies.

Median kisspeptin levels were 2822 pmol/L (IQR 1913-0.48; n=33), 3953 pmol/L (2823-5615; n=31), 4545 pmol/L (3182-6182; n=30) and 3711 pmol/L (2546-4937; n=26) at antenatal visits 1-4, respectively (Fig. 39B). There is considerable overlap of kisspeptin levels as gestation advances but significant increases are noted between visits 1 and 2 ($p<0.05$), visits 1 and 3 ($p<0.001$), visits 1 and 4 ($p<0.001$) and visits 2 and 4 ($p<0.01$) (Fig. 39B). The difference in kisspeptin levels between sequential visits was analysed and while an increase overall is seen in kisspeptin levels from visit 1-3, there is a decrease in levels from visit 3 to 4. Between antenatal visit 1 and 2 the median increase in kisspeptin is 1311 pmol/L (IQR 285.9-2389; n=32), between antenatal visits 2 and 3 the median increase is 620 pmol/L (IQR -7.1-1343; n=24), between antenatal visits 3 and 4 the median decrease in kisspeptin is -1135 pmol/L (IQR -2073—578.4; n=26). Significant decrease is noted between the difference in levels between visits 1-2 and 3-4 ($p<0.0001$), and visits 2-3 and 3-4 ($p<0.01$).

Three participants (6, 9 and 22) developed pre-eclampsia in the 3rd trimester of their pregnancy. Median FAVs were 0.19 cm^3 (IQR 0.1-1.32; n=3), 0.24 cm^3 (0.21-0.44; n=3), 5.03 cm^3 (0.95-9.11; n=3) and 4.81 cm^3 (1.67-7.95; n=3) at antenatal visits 1-4, respectively. Median kisspeptin levels in this small sub-group were 1624 pmol/L (IQR 1680-2383; n=3), 3273 pmol/L (1098-3718; n=3), 2993 pmol/L (1397-4924; n=3) and 3427 pmol/L (2883-3971; n=3) at antenatal visits 1-4, respectively (Fig. 41A). Due to the small sample size, further sub-group analysis was not conducted.

Two participants (18 and 30) delivered preterm infants. Only one measurement of FAV (0.49 cm^3) and one measurement of kisspeptin (3437 pmol/L) were obtained for participant 18 at visit 1, as she subsequently delivered at 26.8 weeks gestation. The FAVs recorded for participant 30 were 1.17 cm^3, 0.25 cm^3 and 0.8 cm^3 at antenatal visits 1-3, respectively. The kisspeptin levels at antenatal visits 1-3 were 1793 pmol/L, 3952 pmol/L and 2568 pmol/, respectively. She subsequently delivered at 33.5 weeks gestation (fig. 42). There was no striking difference between maternal serum levels of hCG or PlGF in these 2 participants and the rest of the cohort. Due to the small sample size, further sub-group analysis was not conducted.

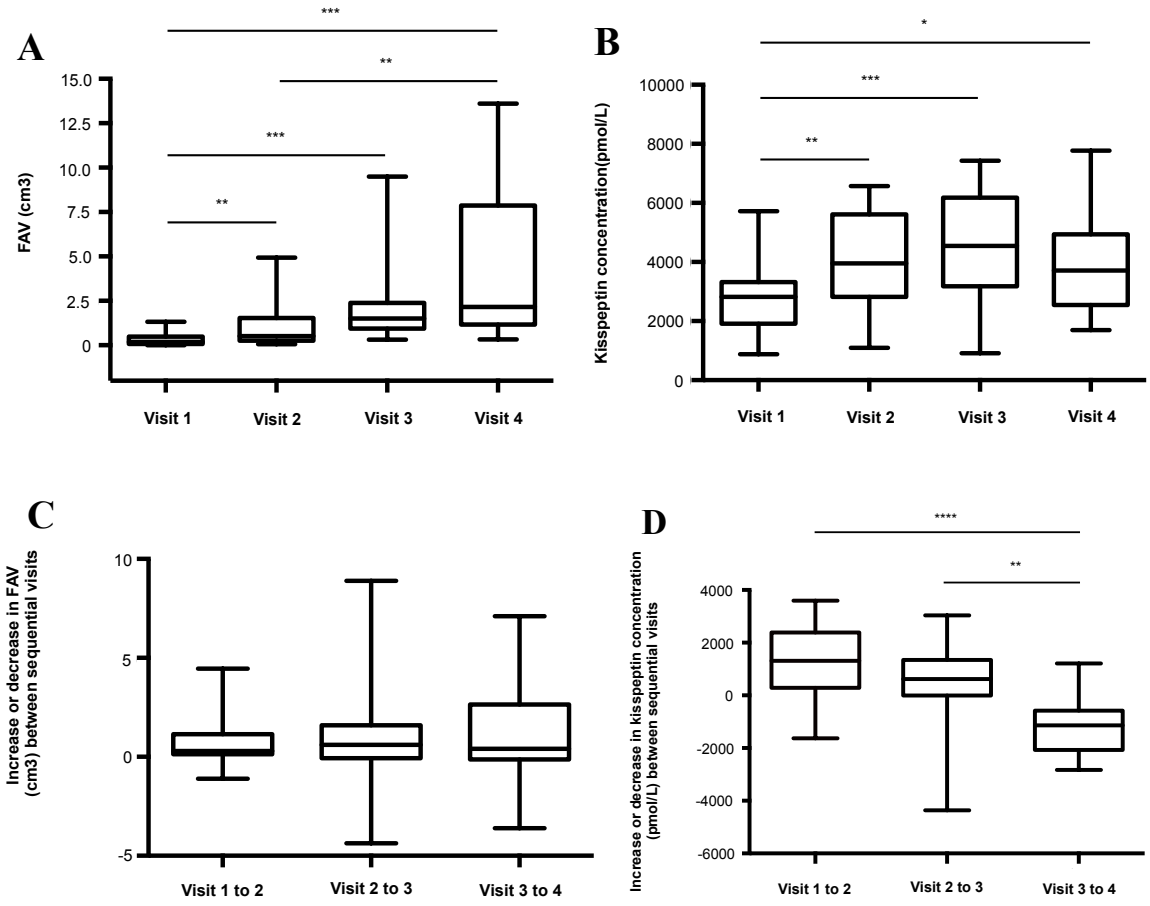


Fig. 39. Foetal adrenal gland volumes and kisspeptin levels at four antenatal visits

Box and whisker plots (A) of foetal adrenal gland volumes (FAV; cm^3) and (B) Maternal serum kisspeptin (KP) levels at the 4 antenatal visits (visit 1, 19-20 weeks; visit 2, 26-28 weeks; visit 3, 34-35 weeks; visit 4, 37-40 weeks). Box and whisker plots of (C) the difference in FAV (cm^3) and (D) Maternal serum kisspeptin levels between sequential antenatal visits. Box plots show the median, upper and lower quartiles and interquartile range (IQR). *p<0.05; **p<0.01; ***p<0.001; ****P<0.0001.

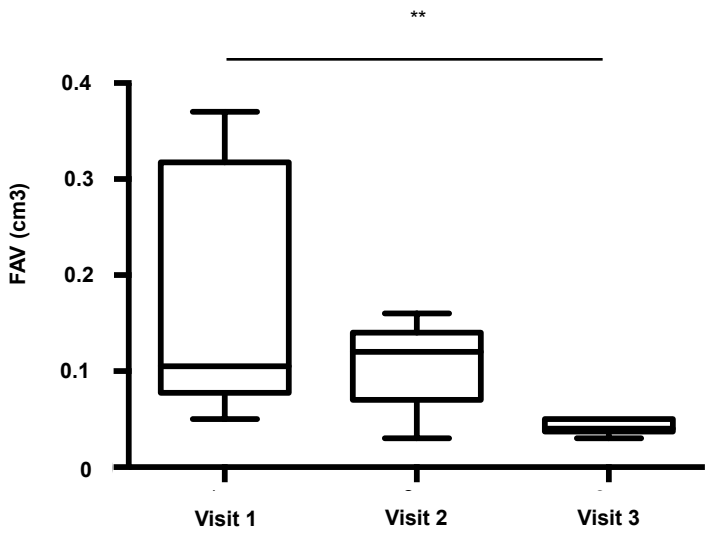
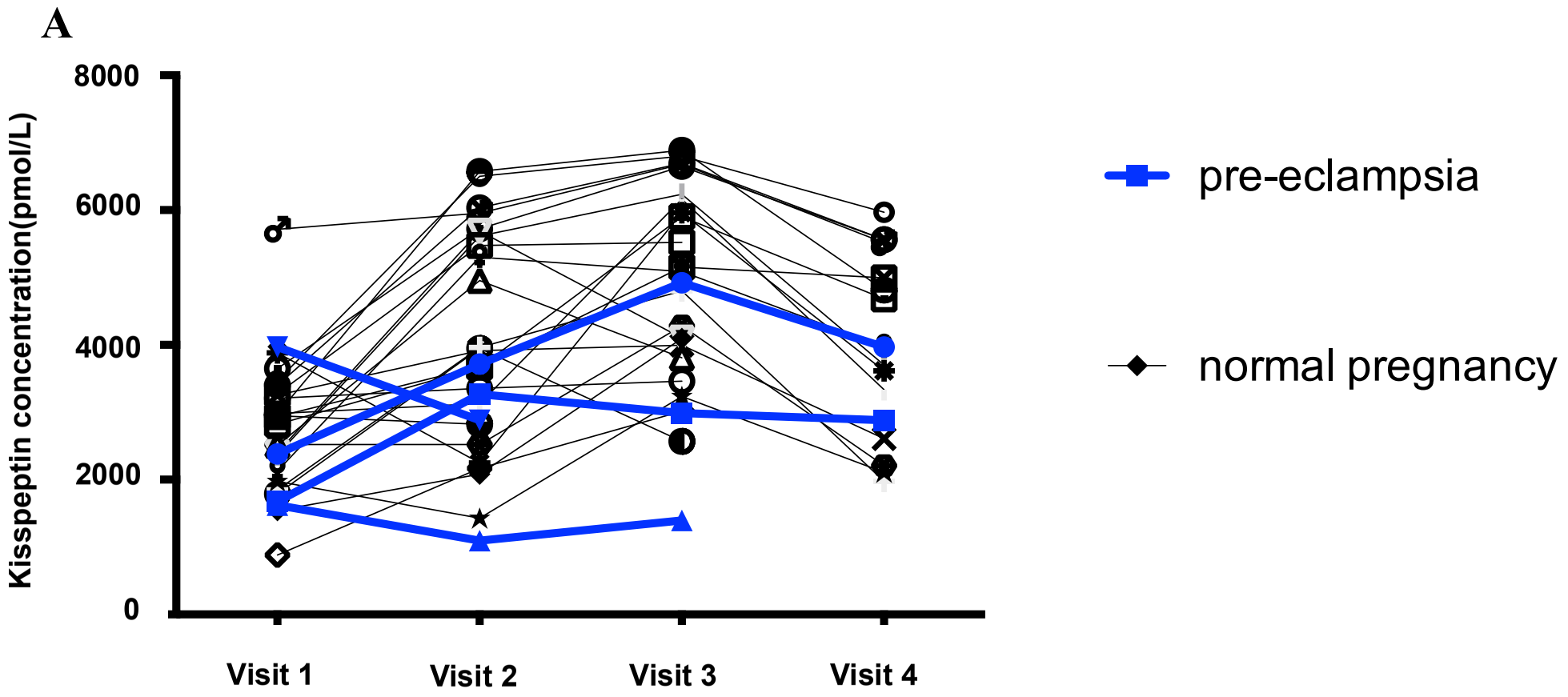


Fig. 40. Postnatal neonatal adrenal gland involution. Box and whisker plots of neonatal adrenal gland volumes (FAV; cm^3) at 3 postnatal visits (visit 1, day 1 of life; visit 2 day 5-7 of life; visit 3 6-8 weeks of life). Box plots show the median, upper and lower quartiles and interquartile range (IQR). **p<0.01.


A
8000
6000
4000
2000
0
Kisspeptin concentration(pmol/L)
Visit 1
Visit 2
Visit 3
Visit 4
pre-eclampsia
normal pregnancy

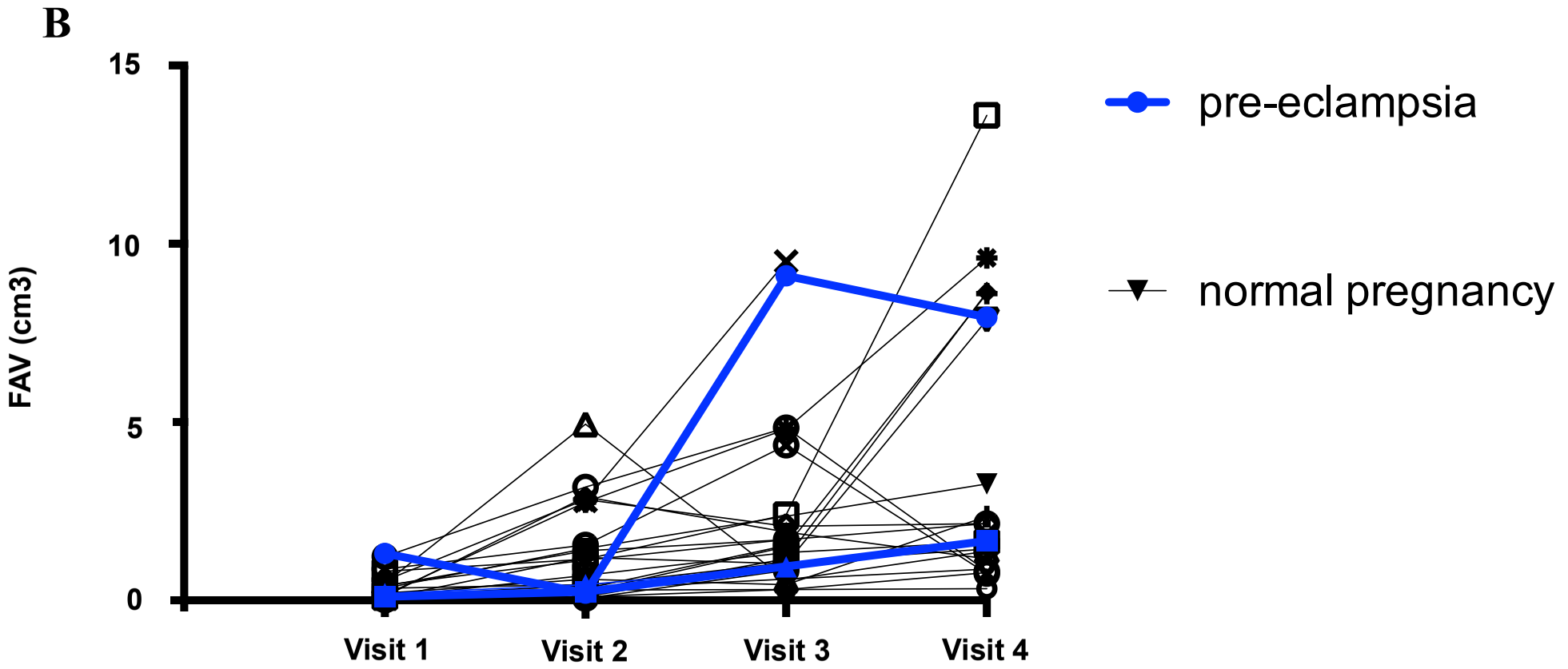


Fig. 41. Sequential maternal kisspeptin levels (A) and foetal adrenal gland volumes (B) for n=33 participants at four antenatal time points. Three participants developed pre-eclampsia (blue).

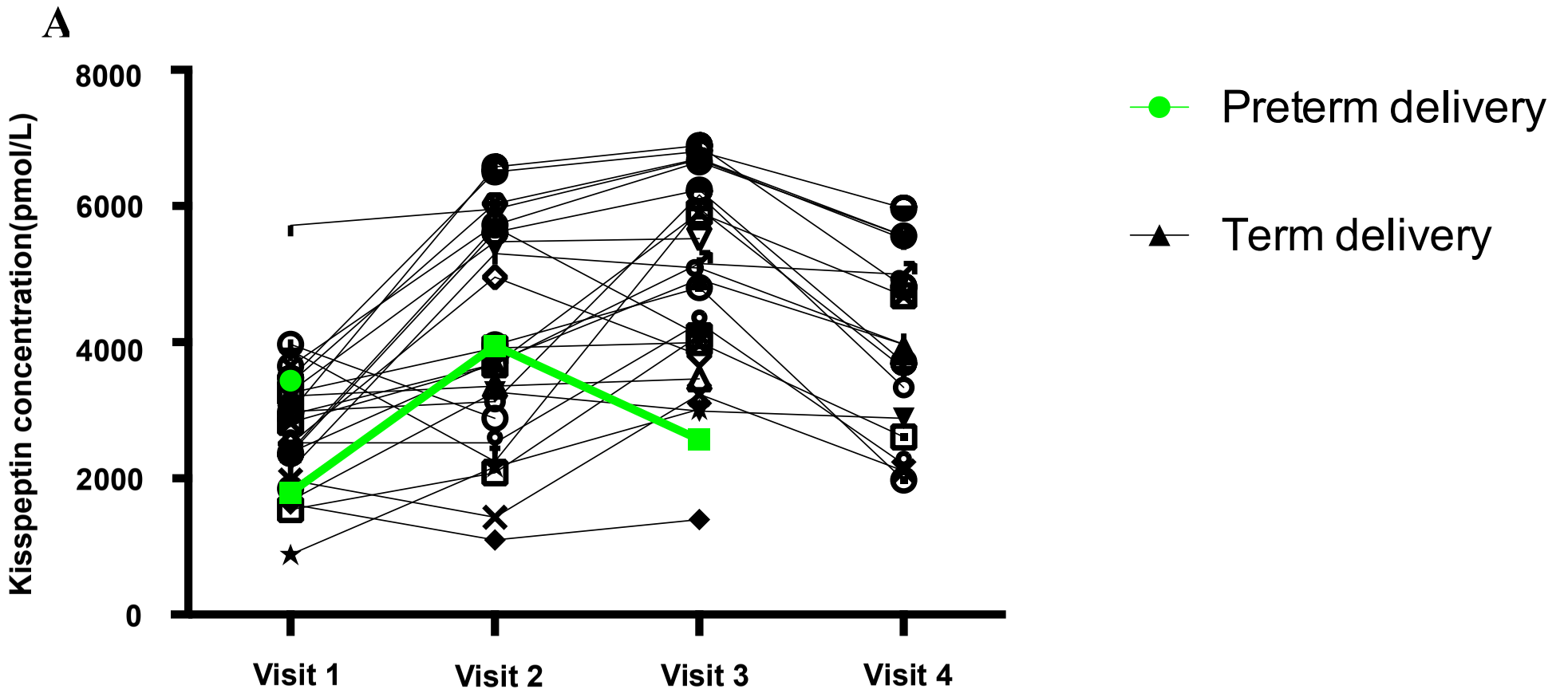


Fig. 42. Sequential maternal kisspeptin levels (A) and foetal adrenal gland volumes (B) for n=33 participants at four antenatal time points. Two participants delivered pre-term (green).

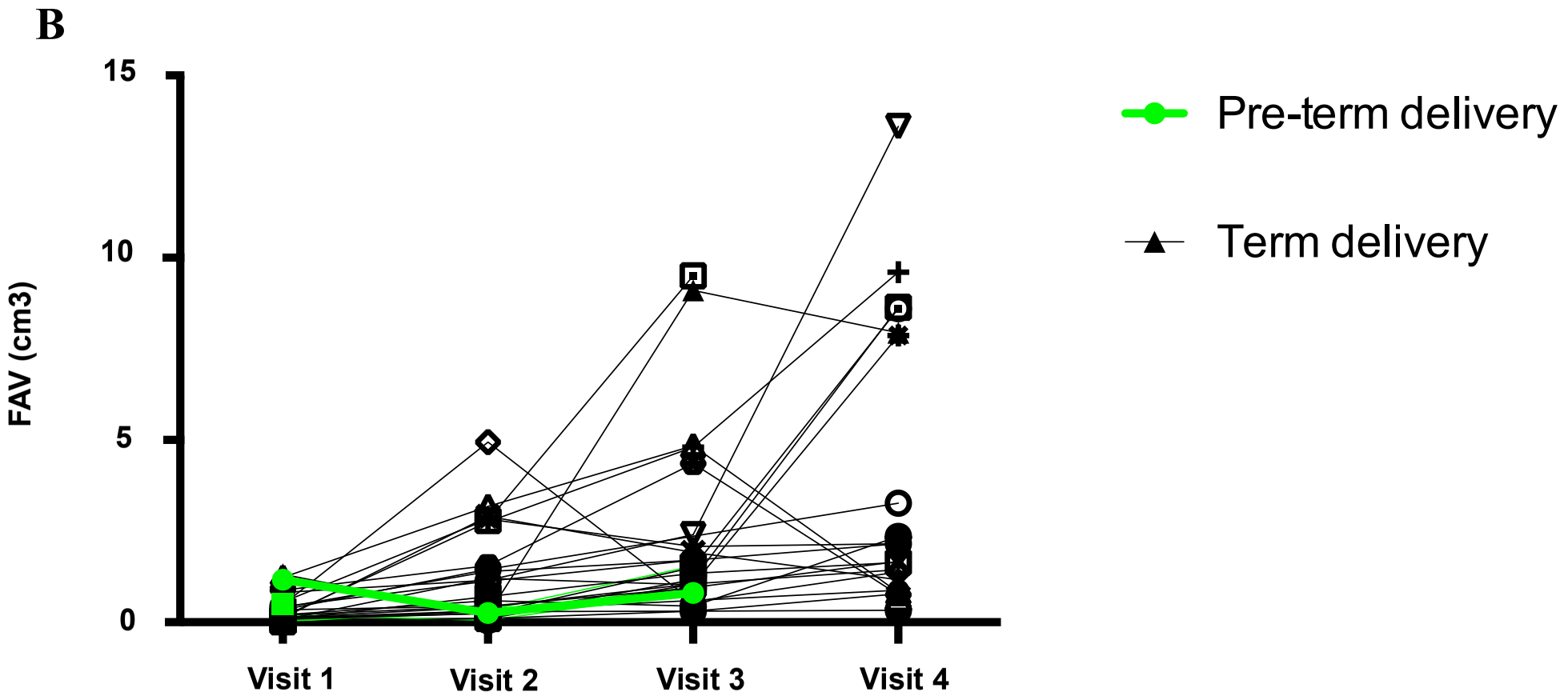


Fig. 42. Sequential maternal kisspeptin levels (A) and foetal adrenal gland volumes (B) for n=33 participants at four antenatal time points. Two participants delivered pre-term (green).

5.4 Relationship between foetal adrenal volume and plasma kisspeptin in singleton pregnancies

To corroborate the *in vitro* data, I assessed the association of the maternal kisspeptin levels with the subsequent FAV increment at the four different time points. The statistical analysis was performed by Dr Sumana Chatterjee (Fellow in Paediatric Endocrinology). The mean increase in FAV between antenatal visits 1 and 2 correlated with the kisspeptin level at visit 1 (r = 0.41, P = 0.026) (Fig. 43) suggesting that the kisspeptin levels between 19 and 20 weeks' gestation (17 to 18 wpc) may influence FAV increase between 19 and 28 weeks' gestation (17 to 26 wpc).

There was no significant difference in the mean rise of FAV between the first and second antenatal visits in male 0.83 ± 0.94 (n = 18) and female 1.28 ± 1.38 (n = 12) infants, respectively (P = 0.30; 95% CI, −0.42 to 1.31). There was no significant correlation between FAV and estimated foetal weight (efw) between the first and second antenatal visits (r −0.166; P = 0.36). Therefore, the significant correlations between maternal kisspeptin and FAV were independent of foetal sex and efw, suggesting that kisspeptin may be important for FA development in mid-pregnancy.

There was no significant correlation between the maternal kisspeptin levels and the subsequent FAV increment at the other antenatal time points. There was no significant correlation between maternal hCG or PlGF at visits 1 or 2 and the subsequent FAV increment. There was no significant correlation found between kisspeptin and hCG or PlGF

at the 4 time points examined, although a complete data-set for each participant was not always available, limiting the analysis.

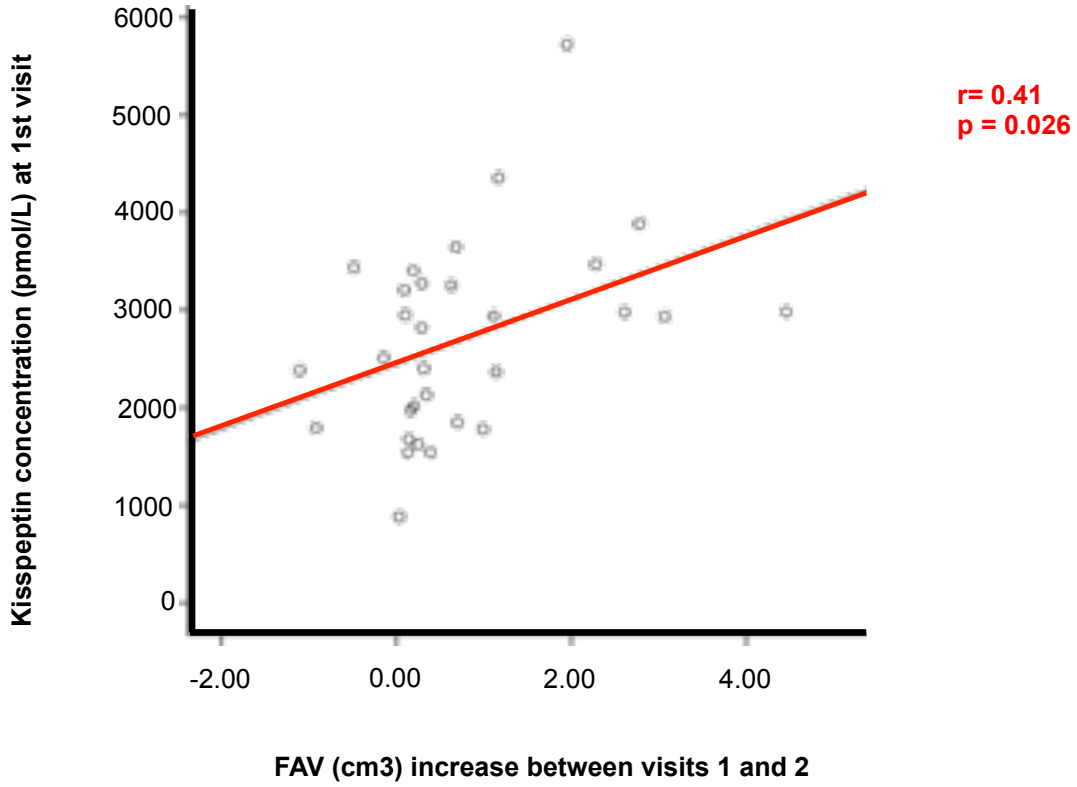


Fig. 43. Foetal adrenal gland volumes and kisspeptin levels at the 1st and 2nd antenatal visits.

Scatter Plots showing (A) the correlation between the kisspeptin (KP) level at the 1st visit and the increase in FAV between the 1st and 2nd antenatal visits. r, pearson coefficient.

5.5 Discussion: Clinical study

3-D ultrasonography (3DUS) is an established and accurate method of assessing foetal organ volumes. More recently it has been reported as a reliable technique to measure HFA volume, with good intra- and inter-observer repeatability [178,209]. A previous cross-sectional study reports correlations between FAV and estimated foetal weight (efw) and FAV and

gestational age (GA) [210]. Interestingly, my data suggest that FAV was independent of both GA and efw. This discrepancy may be explained by the fact that the data was longitudinal and is therefore more likely to show true correlations. Chang et al observed larger FAVs than those obtained in the current study [210] and two other groups report slightly lower FAVs at comparable GAs [178,209]. These differences may be attributed to different methodologies and inclusion criteria employed. Additionally, all three studies report cross sectional rather than longitudinal data. Importantly, the FAV measurements in the current study are in agreement with data obtained from a detailed postmortem study [211].

Circulating kisspeptin concentrations increase dramatically during pregnancy and its levels reflect the amount of viable placental tissue [158]. Consequently a decline in the levels may be associated with increased miscarriage and preeclampsia [160,163]. This was also demonstrated in twin pregnacies where the death of one twin was associated with lower kisspeptin levels [163]. Serial measurements of plasma kisspeptin in pregnant women have not previously been undertaken but cross-sectional data suggest that the levels increase as pregnancy progresses [158]. I found a significant increase in circulating kisspeptin in pregnant females between 20 and 28 weeks gestation which correlates with the second trimster rise in FAV. This increase in FAV is independent of sex and efw. It also coincides with the *in vitro* data, which shows a significant increase in *Kiss1R* mRNA expression in second trimester (13-22 weeks) HFA cells, and DHEAS production from mid-trimester (10-22 gestation) HFA cells following kisspeptin treatment.

Abnormal activation of the foetal HPA axis and enlargement of the FAV has been associated with impending preterm birth [178,212]. The onset of parturition is likely to involve a complex interplay between placental hormones. Intriguingly, my clincial study of serial

measurements of kisspeptin levels revealed a general slight decline late in the 3rd trimester between ~34 weeks and 38-40 weeks gestation, prior to parturition. This has not been previously reported and may concord with my *in vitro* data which showed that CRH and kisspeptin together decrease DHEAS production. The relative concentrations of both placental hormones may be extremely critical to the timing of parturition. Kisspeptin falling towards the end of pregnancy and CRH rising from 35 weeks could be postulated to result in a sharp rise in DHEAS production and hence oestrogen. The surge in oestrogen converts the in utero environemnt to a contractile state, preparing for expulsion of the foetus. CRH is proposed to play critical roles in foetal maturation and the onset of parturition as the levels of CRH increase as pregnancy progresses and peak from 35 weeks gestation corresponding with a fall in the level of cortisol binding protein [105]. It is possible that kisspeptin modulates the effects of CRH in mid gestation however, its role in late pregnancy when change in CRH levels is critical, warrants further investigation.

5.5.1 Limitations and future studies

This study was designed to capture two cohorts of women (uncomplicated normal pregnancy vs pregnancy later complicated by pre-eclampsia) that would be serially followed up from ~20 weeks gestation. As had been intended in the original study design, this work would be usefully extended by the inclusion of patients with pre-eclampsia. Unfortunately during the time scale of this project which included an extension to the recruitment period, the projected numbers of participants who developed PE did not maerialise. This may reflect a lower prevalence of PE (1.3%) in the local population compared to a few years ago with the initial screening pilot study was undertaken. It may also be the case that the

screening method utilised (uterine artery Doppler measurements at 12 weeks) is not as sensitive or specific as the pilot data had indicated.

A very small number of participants developed PE or delivered spontaneously preterm. Due to the small sample size it was not possible to conduct meaningful subgroup analysis. Larger scale clinical studies would be needed in order to ascertain outcomes of pregnancies associated with abnormal kisspeptin-Kiss1R signalling. Limitations to measuring kisspeptin currently, utilising the method described in this study (mainly processing complexity and expense of the test) does have implications however for its use in larger clinical trials.

Further extensions to this clinical study would include simultneous measurements of maternal oestrogens in blood or urine (specifically oestriol as a marker of foetal adrenal steroidogenesis); ideally these should be measured in a normal cohort of pregnant women and a cohort with placental dysfunction (and hence presumed lower levels of kisspeptin) as this would be highly informative. Plasma concentrations of oestrone, oestradiol, and oestriol increase as human pregnancy progresses, with daily excretion rates at term approximating 2, 1, and 40 mg, respectively [213,214]. The assessment of foetoplacental function by measurement of maternal oestrogens has been used previously [215]. Low serum oestradiol concentrations during the first trimester increase risk of spontaneous miscarriage [216]and low levels in the third trimester are associated with poor obstetric outcome [217]. The causes of low oestrogen excretion may be due to failure of foetal steroidogenesis or in placental dysfunction, or both, however it has previously been demonstrated that abnormal foetal steroidogenesis rather than reduced placental metabolistm is the most common cause of low oestrogen excretion of unknown aetiology [218]. Taken together, these observations

suggest that oestrogen plays a critically important role in the maintenance of human pregnancy.

Progesterone and oestrogens are antagonistic in the parturition process [219]. Progesterone promotes uterine quiescence by producing uterine relaxation, stabilizing lysosomal membranes and inhibiting prostaglandin synthesis and release [220]. By contrast, oestrogens destabilize lysosomal membranes, ripen the cervix, and increase the sensitivity of the myometrium to oxytocin by augmenting prostaglandin biosynthesis. Interestingly, it would appear that in human pregnancy, oestrogen is produced in considerable excess, suggesting interplay between factors to maintain balance between drivers that cause uterine quiescence and those that produce contractility and uterine emptying.

If time, resources and cost permitted, ideally this study would have incorporated serial masurement of other maternal hormones, such as CRH and ACTH, as well as progesterone. Serum had been taken and saved with this intention as these data would provide a great deal of information on the interplay *in vivo* between factors regulating foetal adrenal development and the role these may play in the onset of parturition.

The aim of this work overall was to improve understanding about normal human adrenal development.

It is hoped that this project will inform future studies into abnormalities of adrenal development and function. Furthermore, postnatal remodeling of the neonatal adrenal and how this impacts on adaptation to life ex-utero is incompletely understood. In our cohort of

infants, serial US measurements of the adrenal were carried out to document this process. A significant decrease in adrenal volume was seen between the first day and the 6-8th week of life. In parallel, urine had been stored for analysis of steroid metabolites by LC-MS/MS with the aim of documenting functional change over time. As previously stated, due to time and resource pressures on a clinical service these samples would be processed as part of ongoing collaborative studies. Involution of the adrenal would therefore be documented in terms of hormone secretion and in adrenal size.

6 General Discussion and Future Work

6.1 Kisspeptin in pregnancy

Proteolytic processing of the full-length 145 amino acid KP protein results in shorter fragments of the molecule with 54 (KP-54), 14 (KP14), 13 (KP-13) or 10 (KP-10) amino acids. Bilban et al showed that first trimester placenta conditioned medium contained KP-10, 13,14 as well as KP-54 [130]. The common feature of all kisspeptins is a carboxyl (C)-terminus region, which is a ten-residue peptide (KP-10) necessary for receptor activation. KP-10 has the highest potency to bind KISS1R and to trigger downstream signalling pathway and in trophoblasts of early placenta, it was shown that only KP-10 was able to increase intracellular Ca^{2+}[130]. To date almost all the data on potential action of kisspeptins over vital parameters of adrenocortical cells, and others such as cancer cells, has been derived from the experiments in which synthetic KP-10 has been used. In pregnancy, it is serum levels of placental-derived kisspeptin-54 that have been measured in studies with reported rises up to 10,000-fold above nonpregnant levels by the third trimester [158]. To have a better understanding of functional activity of the native molecule, it is important to know how the activity of cells is affected by the kisspeptins released from its physiological sources such as placenta. Rasoulzadeh et al analysed the potential effects of placental kisspeptins and synthetic KP-10 on proliferation, adhesion, invasion, migration, and pro-inflammatory cytokine production in breast cancer cells [221]. Their observations suggest that placental kisspeptins differentially modulate vital parameters of breast cancer cells, possibly through modulation of pro-inflammatory cytokine production. Placental kispeptins were reported to markedly reduce proliferation of breast cancer cells in a dose- and time-dependent manner [221]. This observation may be due to cumulative effects of different placental kisspeptin fragments working in concert. Indeed, activity of the proteins is extensively influenced by their structure and it is highly probable that placental kisspeptins with native conformation are more stable compared to synthetic polypeptides. In support of this hypothesis, their

results showed that placental kisspeptins have higher potency to block cell proliferation and retained its activity until 72 hours, when no anti-proliferative activity for KP-10 was observed [221]. Future extensions of my *in vitro* work therefore would ideally include culture of 1st, 2nd and 3rd trimester placental villous tissue explants. Culture supernantants could then be collected and examined for the content of kisspeptins released from explants and then utilized in experiments with adrenocortical cells.

6.2 The putative role of placental kisspeptin in foetal adrenal steroidogenesis

The mechanisms that regulate DHEAS production by the foetal adrenals are incompletely understood. CRH has been shown to be as effective as ACTH at stimulating DHEAS production, in HFA cells however, it has been shown to be less potent than ACTH at stimulating cortisol production, indicating that its actions are preferentially directed toward increasing DHEAS synthesis [110]. My data indicates that Kisspeptin-stimulated production of DHEAS by primary HFA cells and H295R cells was comparable to stimulation with ACTH or CRH alone. This may be due to the heterogeneity of the SF1-positive cells and further experiments might examine *SF1* RNA and protein levels, *Kiss1R* RNA and protein levels, and DHEAS production of cells treated with kisspeptin vs. ACTH/CRH. Measurement of other steroids including cortisol and aldosterone would also be useful to ascertain whether there is preferential output of androgens with kisspeptin, and if so if this is due to changes in the regulation of steroidogenic enzymes. Kisspeptin may stimulate DHEAS more specifically than ACTH or CRH, for example, by increasing abundance of cytochrome P450 cholesterol side-chain cleavage and 17alpha-hydroxylase/17,20 lyase but not 3beta-hydroxysteroid dehydrogenase in adrenal cells. Alternatively it may alter cell number, indicating that it is mitogenic for foetal adrenal cortical cells. These unanswered questions would form the

basis of future work.

6.3 The putative role of placental kisspeptin in foetal adrenal growth

Kisspeptins appear to be regulators of invasion both in pathological conditions (tumours), and in physiological situations including trophoblast invasion in pregnancy. The rapid development of the foetal adrenal could be viewed as akin to the rapid growth of tumours although this organ is non-invasive. As such clear regulatory mechanisms for growth must exist. The mechanisms by which kisspeptin may regulate adrenal growth have not been studied to date but are likely to involve processes that are emerging in other tissues, such as in tumour biology. For example, kisspeptin has been shown to inhibit matrix metalloproteinase (MMP) expression and activity *in vitro* [222], and MMPs are critically involved in events taking place at the cell–extracellular membrane interface such as cell division, migration and morphogenesis and ultimately regulate cell behavior [223].

Apoptosis appears to occur in the developing HFA cortex [20]. There is evidence of cellular apoptosis, determined by morphological criteria, in the HFA primarily in the central portions of the FZ [23] and studies have shown detection of apoptotic cells by *in situ* analysis of DNA fragmentation and found that the labeling index of apoptotic nuclei is greater in the central areas of the FZ than in the DZ [19]. Kisspeptin was originally discovered from a metastasis tumour suppressor gene, *KISS1* [117] and it has been suggested that kisspeptin may suppress metastasis by induction of apoptosis in cells, since metastatic spread is dependent on cell growth [224]. However reports in the literature have been conflicting. Culturing recombinant Chinese hamster ovary cells with KP-10 was shown to have a strong anti-proliferative effect, while no evidence of apoptosis was detected [119]. By contrast, in a more recent study using

human mammary carcinoma cells, stimulation of KISS1R by KP-10 caused up-regulation of several genes involved in cell cycle progression (including cyclin dependent kinase inhibitor 1A (*CDKN1A),* Growth Arrest and DNA damage-inducible 45 *(GADD45A, GADD45B)*, and Hypoxia-inducible factor-1α-responsive DNA-damage-inducible transcript 4 (*HIF1A*-responsive *DDIT4*)), cell cycle arrest and apoptosis [125]. Interestingly a study by Ziegler et al [225]showed a connection between the antiproliferative effect of kisspeptin and the nature of KISS1R expression, with evidence for KISS1R mediated cellular mechanisms involved in proliferation only detectable in cells with up-regulated receptor expression. My results show decreased expression of Kiss1R at mRNA and protein level at supraphysiological concentrations of kisspeptin. *In vivo* the dramatic fold-change in kisspeptin concentration through gestation may result in changes in Kiss1R expression in foetal adrenal cells in a spatio-temporal manner, hence effecting a change in the postulated effects of kisspeptin on cells. Further experiments in the H295R cell line and HFA cells would seek to examine whether kisspeptin has a proliferative or anti-proliferative effect these cells expressing Kis1R endogenously.

The rapid HFA growth is almost entirely due to enlargement of the FZ, and in contrast to the DZ, mitotic figures in the FZ are scant. Collectively, the FZ appears to grow by hypertrophy under limited cell proliferation [20]. In the foetal rhesus monkey, it has been demonstrated that growth of the FZ occurs primarily by hypertrophy, in response to increased endogenous ACTH secretion provoked by metyrapone treatment [226]. It is unknown whether kisspeptin treatment of FZ cells would also result in hypertrophy.

Additionally, the HFA is one of the most highly vascularized organs in the human foetus [20]. The development of an extensive vasculature in this organ is essential for organ growth and

delivery of tropic agents and steroid hormone precursors to the gland and secretion of hormone products into the peripheral circulation. Angiogenesis, the formation of new blood vessels from pre-existing vascular beds, is considered an integral process for organ growth. Because the HFA undergoes a phase of rapid growth in midgestation, angiogenesis likely is essential for the rapid growth of the HFA. Angiogenesis is a common feature of both implantation and cancer spread [227] and is of vital importance in establishing the placenta. Kisspeptin and KISS1R expression have been reported in human aorta, coronary artery and umbilical vein and kisspeptin acts as a vasoconstrictor (a crucial phase in angiogenesis) in isolated coronary arteries [129], suggesting a role in the vascular system. A potential angiostatic effect of kisspeptin may also explain, at least in part, the anti-metastatic potential of this peptide given that angiogenesis is intimately involved in this process. Detailed *in vitro* and *in vivo* studies to investigate the potential angiostatic effects and to determine the molecular mechanisms underpinning such effects of kisspeptin are required.

6.4 New frontiers and challenges

Advances in foetal imaging, genomics, transcriptomics, proteomics, metabolomics, and minimally invasive techniques, as well as a better understanding of the intra-uterine endocrine milieu and natural history of many diseases have revolutionized the management of maternal, foetal and neonatal conditions in the last decade. The potential role of kisspeptin in the promotion of growth and functional development of the foetal adrenal is an area for much future research. Furthermore, the roles this unique organ plays during pregnancy as part of the very transient but critical foeto-placental unit require definition.

The future translational aspects of this work with respect to the potential role of the foetal adrenal in the maintenance of a pregnancy, are also important. The mechanisms and foeto-placental interactions that enable maintenance of pregnancy and survival to term remain unclear, as reflected by rising rates of spontaneous preterm birth over the past 20 years (Office of National Statistics (released October 2011) http://www.ons.gov.uk). In 2009 in England and Wales ~8% of babies (1 in 13) were born prematurely.

The integration of various technologies combined with computational and mathematical models could be used to identify new therapeutic agents, drug targets, and novel biomarkers for at risk-pregnancies, as demonstrated for other paradigms [228–230]. Computational models integrate quantitative data from complex systems and could be used as platforms to investigate the dynamic biochemical properties of cells. Studying the dynamics of the activity of a pathway may provide prognostically relevant information that differs from the information provided by other biomarkers, due to their static nature [231]. Therefore, due to the complexity of the various interacting pathways involved in the regulation of adrenocortical function, and the dynamic partnership between the foetal adrenal and the placenta, it would be interesting to develop similar models to explore the role these play in pregnancy and the timing of parturition [85].

Preterm birth is one of the greatest threats to the developing foetus and a major cause of perinatal morbidity and mortality. It is evident that the foetal adrenal cortex, together with the placenta forms a unique foeto-maternal endocrine unit that regulates oestrogen production during development, and is modulated by placental regulators. Future studies may be warranted, examing the role of kisspeptin as a potential novel biomarker for at-risk

pregnancies, as these may reveal targets for therapeutic agents which could arrest threatened spontaneous preterm birth.

6.5 Conclusion

Kisspeptin-Kiss1R signalling may be a key regulator of HFA development and steroidogenesis and therefore an integral component of the foeto-placental unit. There is scope for more research into further defining the underlying molecular mechanisms involved in the control of foetal adrenal development by kisspeptin. As well as being critical in the regulation of placentation in early pregnancy, kisspeptin may have a key physiological role in intrauterine homeostasis and the maintenance of pregnancy, particularly in the second trimester. There is a need to further evaluate the role of kisspeptin in the third trimester, particularly with respect to adverse outcomes of pregnancy, and this information may help in directing future patient surveillance. Therefore, overall this data suggests a novel functional role for kisspeptin *in utero*.

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APPENDIX 1: ETHICAL APPROVAL BRIGHTON AND SUSSEX NRES COMMITTEE

NHS
Health Research Authority
NRES Committee South East Coast - Brighton and Sussex
Health Research Authority
Ground Floor, Skipton House
80 London Road
London
SE1 6LH

Telephone: 020 797 22565
Facsimile: 020 797 22592

18 December 2012

Dr Harshini Katugampola
Clinical Research Fellow
Queen Mary University of London
Centre for Endocrinology
Charterhouse Square
London
EC1M 6BQ

Dear Dr Katugampola

Study title:	**The Control of the Feto-Placental Unit by Kisspeptin and Corticotropin Releasing Hormone (CRH): Potential novel Insights into the pathogenesis of premature birth.**
REC reference:	**12/LO/1755**
Protocol number:	**1**
IRAS project ID:	**109533**

Thank you for your letter of 20 November 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mrs Nischinth Cherodian, NRESCommittee.SECoast-BrightonandSussex@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Evidence of Insurance or Indemnity		30 July 2012
Investigator CV		10 October 2012
Letter from Sponsor		11 October 2012
Other: Letter to GP	1	10 July 2012
Other: Peer Review		19 July 2012
Other: CV of Helen Storr		16 October 2012
Other: CV of Peter King		18 October 2012
Other: CV of Leo Dunkel		
Participant Consent Form: Parent	1	13 November 2012

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

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Other: CV of Helen Storr		16 October 2012
Other: CV of Peter King		18 October 2012
Other: CV of Leo Dunkel		
Participant Consent Form: Parent	1	13 November 2012

Yours sincerely

Dr Simon Walton
Chair

Email:NRESCommittee.SECoast-BrightonandSussex@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Gerry Leonard*
(sponsor and lead R&D)

KYS

UNIVERSITY OF EASTERN FINLAND

MATERIALS TRANSFER AGREEMENT

This agreement is made on the 10th of October 2012

BETWEEN

(1) **Kuopio University Hospital** whose principal administrative offices are at P.O.Box 1777, 70211 Kuopio, Finland (hereinafter called **"The Provider"**) and

(2) **Queen Mary and Westfield College, University of London** (hereinafter called "The Recipient") whose principal place of business is at Mile End Road, London E1 4NS, U.K. (hereinafter called **"The Recipient")**

WHEREAS

(A) The Provider has developed and owns, or is beneficial owner of, certain materials and information, patent rights and other intellectual property relating to the said materials and information.

(B) The Recipient wishes to obtain samples of the said materials and background information from The Provider for the purpose of an in-house research project only.

(C) The Provider agrees to provide the materials and information on the terms set out below.

NOW IT IS HEREBY AGREED AS FOLLOWS

1. DEFINITIONS AND INTERPRETATION

1.1 In this agreement the following words and phrases shall have the following meanings unless the context requires otherwise:

(a) "Commencement Date" shall mean the day after the date of signature of this Agreement by both parties

(b) "Materials" shall mean all materials provided by The Provider, including any progeny or unmodified derivatives thereof, as described in Schedule 1 of this agreement and which may from time to time be amended to written agreement between the parties.

(c) "Scientist" shall mean the Recipient Scientist.

(d) "Field" shall mean the use of the Materials by the Recipient for a research project as set out in Schedule 2 of the Agreement.

2. USE AND STORAGE OF MATERIALS

2.1 Upon execution of the Agreement, The Provider agrees to supply the Materials and Information which shall be used solely within the Field.

2.2 The Materials shall be used for non-commercial research purposes only. The Recipient will not manufacture, sell or sub-license for manufacture and sale upon a commercial basis, the Materials. It is expressly understood by the Recipient that the Materials will at all times be used in accordance with applicable laws and regulations. It is the sole responsibility of The Recipient to ensure that all relevant approvals and authorisation for the proposed research are obtained.

2.3 In the event where human tissue or products of human origin are implied by this agreement, The Recipient and The Provider undertake to comply with the regulations related to human tissue or products of human origin to obtain all necessary approvals prior to the transfer of such human tissue or products of human origin.

2.4 The Recipient shall dispose of any unused Samples comprising human tissues in accordance with the UK Human Tissue Act 2004 or The Recipient shall return all unused/residual Samples to the Provider at the end of the study, or at the Termination of this Agreement if sooner.

2.5 The Recipient shall ensure that access to the Materials is restricted to The Recipient and its authorised employees and agents, who have reasonable need to access such Materials pursuant to the Study and who have been informed of The Provider's interests in the Materials and Confidential Information and the terms of this Agreement.

2.6 The Materials shall at all times remain the property of The Provider and The Recipient shall not pass any of the Materials to a third party without the prior express written approval from The Provider.

2.7 In the event that The Recipient wishes to exploit or use any Materials on a commercial basis then they will make a formal written request to The Provider seeking whether, at the discretion of The Provider a license may be granted, and upon what terms such a license may be granted.

2.8 Nothing contained herein shall be construed as granting any license under any Intellectual Property rights vested in The Provider or any right to use the Materials or any information other than herein expressly specified.

2.9 The Recipient acknowledges that any and all Intellectual Property Rights subsisting in or used in connection with the Materials are and shall remain the property of The Provider; The Recipient shall not during or after expiry or termination of this Agreement in any way dispute the ownership thereof by The Provider.

3. CONFIDENTIALITY AND PUBLICATION

3.1 Subject to section 3.2 the Recipient agrees not to transfer, transmit or in any other way disclose the materials or any related information to any third party without the written consent of The Provider.

3.2 In the event that the Recipient wishes to publish details of the research project in scientific journals, the Recipient will be free to publish the results of the research project using the Materials on condition that The Provider is supplied with a copy of any such results 30 days prior to submission of such publication and providing that The Provider is acknowledged as the supplier of the Materials used in said published research. The Provider may request the approval, deletion or disguising of any Confidential Information relating to Provider patients or the activities of the Provider but such approval shall not unreasonably be withheld or delayed.

3.3 Each party agrees not to use or refer to this Agreement in any promotional activity, or use the names or marks of the other without express written permission. However, this paragraph shall not preclude either party's attribution of authorship in, and distribution of, academic literature reporting the results of research conducted with the Materials.

3.4 The obligations of confidence referred to in this Clause 3 shall not extend for more than 3 years after information is provided under this agreement and shall not extend to any information which:

(a) is or becomes generally available to the public otherwise than by reason of a breach by The Recipient party of any provision of the clause 3.
(b) is known to The Recipient party and is at its free disposal prior to its receipt from the other.
(c) is subsequently disclosed to the recipient party without obligations of confidence by a third party owing no such obligations to the disclosing party in respect thereof.
(d) is required to be declared by law, or
(e) is inadvertently developed by The Recipient personnel without access to The Provider's information.

4 WARRANTIES/LIABILITIES

4.1 Subject to Section 3.2 the Recipient warrants that the Materials and related information will only be disclosed to, and used by, Scientists working on the research project.

4.2 Both parties understand and agree that the Materials are provided without warranty of merchantability or fitness for a particular purpose or any other warranty, express or implied and without any representation or warranty that the use or supply of the Materials will not infringe any patent, copyright, trademark or other right.

4.3 In no event shall The Provider be liable for any use by the Recipient of the Materials transferred under this Agreement. The Recipient agrees to indemnify and hold harmless The Provider for any loss, claim, damage, or liability of whatsoever kind or nature which may arise from, or in connection with, this Agreement or the use, handling or storage of the Materials by the Recipient.

5 TERMS

The term of this Agreement is 2 years from the date of execution. The obligations of both parties under Clauses 3 and 4 of this Agreement shall survive termination of this Agreement for any cause.

6 GOVERNING LAW AND JURISTRICTION

The validity, construction and performance of this Agreement shall be governed by the English law and English courts shall have exclusive jurisdiction in a matter pertaining to this agreement.

SPACE BELOW LEFT INTENTIONALLY BLANK

IN WITNESS WHEREOF the parties hereto have executed this document the day and year first before written

For and on behalf of
The Provider

Signed

ULLA SAMKLINGER

Print Name

Consultant Neonatologist, Head of the NICU, Kuopio University Hospital

Title

14th October 2012

Date

For and on behalf of
The Recipient

Signed

Print Name
Graeme Brown

Title
Director Technology Transfer

31/10/2012

Date

Acknowledged by the Principal Investigator/Scientist (Employee of the Recipient)

Signed

Dr Helen Storr
Print Name

Senior Lecturer and Honorary Consultant in Paediatric Endocrinology
Title

28/10/2012
Date

Schedule A

Human fetal adrenal (HFA) tissue collected following post-mortem from the pathology department archive at the University of Kuopio (for immunohistochemistry and immunofluorescence studies).

In total it is anticipated that a minimum of 40 samples over 18 months will be required for this project.

Anonymised clinical data collected from maternal records and records of preterm and term infants.

Schedule B

The Materials will be used by The Recipient for a research project:

Research Project Title:

The Control of the Feto-Placental Unit by Kisspeptin and Corticotropin Releasing Hormone (CRH): Potential novel insights into the pathogenesis of premature birth.

Ethics Approval Reference No.: Ethical approval pending

Background

During pregnancy, the human fetal adrenal (HFA) cortex grows due to enlargement of the inner fetal zone (FZ). HFA growth is paralleled by increased steroidogenesis, which enables the feto-placental unit to maintain pregnancy.

Placental corticotropin-releasing hormone (CRH) and kisspeptin increase dramatically during pregnancy and their biological roles remain to be fully elucidated. Data from animal models suggest that sonic hedgehog (Shh) signalling plays an important role in adrenal development.

Laboratory studies

This project will be carried out in collaboration with Dr Ulla Sankilampi (Post-doctoral Research Fellow, Consultant in Neonatology). The factors responsible for regulating growth and function of the HFA will be investigated. The spatio-temporal expression of the kisspeptin receptor (Kiss1R) together with CRHR receptor subtypes (CRHR1 and 2) will be examined in HFA tissue from a range of gestations, and the role of Shh signalling in HFA development will be clarified.

The tissue sections will be stained at QMUL (under a MTA) using antibodies against Kiss1R and CRHR1 and 2, and co-stained with antibodies against markers of adrenocortical zones such as anti-CD56 (definitive zone (DZ)), or anti-SULT2A1 (FZ). Co-expression of Kiss1R and CRHR with Shh, a marker of subcapsular progenitor cells, will be investigated using serial *in situ* hybridization (for Shh) followed by immunofluorescence (for the receptors).

Newcastle University

MATERIAL TRANSFER AGREEMENT FOR THE PROVISION OF HUMAN- TISSUE FOR (NON-COMMERCIAL) ACADEMIC RESEARCH

This Agreement is made the 17th day of August 2012 by and between:

The University of Newcastle Upon Tyne an unincorporated charitable organisation established under the Universities of Durham and Newcastle upon Tyne Act 1963, a statute of England, whose address for service is King's Gate, Newcastle upon Tyne, NE1 7RU **("Newcastle")**, through the Human Developmental Biology Resource (HDBR) at the Institute of Human Genetics **(the "Providing Scientists").**

And

Queen Mary and Westfield College, University of London having its principal office at Mile End Road, London E1 4NS, UK **(the "Institution")**.

The Providing Scientists have collected or intend to collect human fetal adrenal and placental tissue **(the "Materials")** as further detailed in Annex 1 and/or as communicated (including by electronic means) by Newcastle to the Institution. The Institution and Newcastle shall maintain competent records of all communications regarding Samples, including identifying numbers, being transferred by Newcastle.

Dr Helen Storr **(the "Recipient Scientist")** who is an employee of **the Institution** located at Charterhouse Square, London EC1M 6BQ, wishes to acquire a Supply of Materials (as defined in the attached Terms and Conditions) for carrying out research The Control of the Feto-Placental Unit by Kisspeptin and Corticotropin Releasing Hormone (CRH): Potential novel insights into the pathogenesis of premature birth. **(the "Project")** as further detailed in Annex 1.

Newcastle is willing to provide human embryonic and foetal materials **(the "Samples")**, should they become available, for the duration of the Project **(the "Term")** on the Terms and Conditions shown below, and the Institution agrees to comply with those Terms and Conditions.

Agreed by the parties through their authorised signatories:

For and on behalf of **NEWCASTLE**	For and on behalf of **INSTITUTION**	Read & Understood by **RECIPIENT SCIENTIST**
Signed	**Signed**	**Signed**
A.C. HALL **Print Name**	GRAEME BROWN **Print Name**	Dr Helen Storr **Print Name**
DI **Title**	DIRECTOR TECHNOLOGY TRANSFER **Title**	
11/9/12 **Date**	30/08/2012 **Date**	17.08.2012 **Date**

APPENDIX 4: MTA (UCL-QMUL)

MATERIAL TRANSFER AGREEMENT

(Which may include the transfer of human tissue)

THIS AGREEMENT is made the 11th of September 2012

BETWEEN

(1) **University College London, Institute of Child Health** ("ICH") whose principal address is 30, Guilford Street London WC1N 1EH, UK; and

(2) **Queen Mary and Westfield College, University of London** ("the Recipient") whose principal place of business is at Mile End Road, London, E1 4NS, UK.

IT IS AGREED as follows:

1 Definitions

1.1 In this Agreement, the following words shall have the following meanings:

Act	Means the Human Tissue Act 2004 as amended from time to time.
Confidential Information	Means all information, including patient, technical, scientific or commercial information, that ICH may provide to the Recipient and that: (a) in respect of information provided in documentary or by way of a model or in other tangible form, at the time of provision is marked or otherwise designated to show expressly or by necessary implication that it is imparted in confidence; (b) in respect of information that is imparted orally, any information that ICH or its representatives informed the Recipient at the time of disclosure was imparted in confidence; (c) all Materials IP; and (d) any copy of any of the foregoing.
Donor	Means the person (if any) from whose body the Material (or any part thereof) has come.
Intellectual Property	Means patents, trade marks, service marks, copyrights, database rights, registered designs, design rights, rights in respect of confidential information, applications for and the right to apply for any of the above, and any similar right

	recognised from time to time in any jurisdiction, together with all rights of action in relation to the infringement of any of the above and any inventions, discoveries and improvements.
Materials	Means all materials as described in the attached Schedule A and includes any and all documents and information provided by ICH and any constructs, strains, portions, progeny and unmodified derivatives (as the case may be) obtained from or as a direct result of the use of such materials; and may include human cells and other "relevant material" as defined in the Act.
Materials IP	Has the meaning given to it in clause 3.4.
Party	Means either ICH or the Recipient; and "Parties" shall mean both of them.
Principal Investigator	Means Dr Helen Storr, who is an employee of the Recipient.
Recipient IP	Has the meaning given to it in clause 3.1.
Research Programme	Means the programme of work to be carried out by the Recipient as described in the attached Schedule B.

2 Supply of Materials

2.1 Upon execution of this Agreement, or as soon as possible thereafter, ICH shall make the Materials available to the Recipient free of charge.

2.2 Delivery of the Materials to the Recipient's laboratory shall be at the Recipient's risk and expense.

2.3 The Recipient shall ensure that the Materials are kept secure at the Recipient's laboratory and ensure that no-one other than the Principal Investigator and the Recipient's authorised employees, who are under the direct and immediate supervision of the Principal Investigator (the "Co-workers"), have access to them.

2.4 The Recipient shall provide ICH with written confirmation of the safe receipt of the Materials promptly after their delivery to the Recipient's laboratory. In addition, the Recipient will inform ICH of the location where the Materials are to be stored and confirm that said facilities are compliant with the Act and any relevant guidance issued by the Department of Health, the Human Tissue Authority or national equivalents.

2.5 If the Recipient's laboratory is not located in England, Wales or Northern Ireland, the Recipient shall ensure, prior to any exportation of the Materials, that the Materials have been and will continue to be handled in accordance with the laws of the Recipient's country.

2.6 The Recipient shall not sell, gift, transfer or otherwise supply the Materials to a third party without the written consent of ICH.

2.7 Under no circumstances shall ICH have any obligation to supply the identity of, or any information that in ICH's reasonable opinion might lead to the identification of, the Donor(s). Each Party agrees that it shall at all times comply with all provisions of the Data Protection Act 1998 as amended from time to time and as applicable to this Agreement.

2.8 The Recipient shall ensure it complies with all applicable laws and any relevant guidance issued by the Department of Health, the Human Tissue Authority or national equivalents,

and all ethical guidelines relating to the use, storage, transportation and disposal of the Materials for research purposes laid down by any competent body or authority. If the Recipient's use of the Materials requires the consent of the Donor, the Recipient shall ensure that the Donor has signed an appropriate consent form and that its use of the Materials is within the scope of that consent form.

2.9 The Recipient shall use the Materials in accordance with good laboratory practice, good clinical laboratory practice and the highest standards of skill and care and shall use the Materials only for the purpose of carrying out the Research Programme. Without limiting the foregoing, the Recipient shall not use the Materials for any commercial purpose or commercially-sponsored research without the prior written consent of ICH even if those purposes are being pursued in any of the Recipient's laboratories.

2.10 Unless otherwise expressly stated in writing, the Materials are intended for research use only and are not to be used for any other purpose including but not limited to, *in vitro* diagnostic purposes, *ex vivo* or *in vivo* therapeutic purposes, investigational use, in foods, drugs or cosmetics of any kind, or for consumption or use in connection with or administration or application to humans or animals.

3 Intellectual Property

3.1 Subject to clause 3.4, any Intellectual Property generated, created or derived by the Recipient from the Materials in the Research Programme, which is not Materials IP, shall belong to the Recipient ('Recipient IP').

3.2 If any Recipient IP and/or Materials IP (as defined in clause 3.4) arises, the Recipient will promptly bring this to the attention of ICH.

3.3 The Recipient hereby grants to ICH a non-exclusive, non-transferable, fully paid-up and royalty-free licence (without the right to sub-licence) to use the Recipient IP for academic research and teaching purposes. Without prejudice to the foregoing, the Recipient would grant ICH an exclusive option, exercisable within 60 days from receipt of notification of any Recipient IP in accordance with clause 3.2, to acquire an exclusive licence (with the right to sub-licence) to use and commercially exploit the Recipient IP on the terms to be mutually agreed between the parties.

3.4 Notwithstanding the provisions of clause 3.1, any Intellectual Property generated, created or derived by the Recipient from the Materials or any part thereof that contains or incorporates the Materials or any part thereof ('Materials IP') shall be owned by ICH. Materials IP shall constitute Confidential Information.

3.5 ICH shall grant to the Recipient a non-exclusive, non-transferable, fully paid-up and royalty-free licence (without the right to sub-licence) to use the Materials IP for the Recipient's academic research and teaching purposes. For the avoidance of doubt, the restrictions and obligations set forth in this Agreement that apply to the Recipient's use, storage and disposal of the Materials shall apply equally to any Materials IP.

3.6 If any commercial revenues result from the Recipient's use of the Recipient IP or otherwise arise from the use of the Materials, ICH shall be entitled to an equitable share of any such revenues that accrue to the Recipient or its successors in title to any Recipient IP. The terms of such revenue sharing shall be amicably settled by the parties taking into account, amongst other factors, the UCL's contribution in developing this IP.

3.7 The Materials and any copies thereof shall remain the property of ICH and the Recipient shall keep the Materials at all times clearly labelled as the property of ICH. No licence under any ICH Intellectual Property is granted or implied by this Agreement.

4 Confidentiality

4.1 As between the Parties, Confidential Information belongs to ICH. The Recipient will not during the term of this Agreement and for a period of 5 years thereafter, disclose to any third party nor use for any purpose except the Research Programme, any Confidential Information.

4.2 The obligations of confidentiality and non-use set out in this Agreement shall not apply to any information that the Recipient can show, by written record: (i) was known to the Recipient before the information was imparted by ICH; (ii) is in or subsequently becomes publicly known through no fault, act or omission on the part of the Recipient; (iii) is received by the Recipient without restriction on disclosure or use from a third party lawfully entitled to make the disclosure to the Recipient without such restrictions; (iv) is developed by any of the Recipient's employees who have not had any direct or indirect access to, or use or knowledge of, the information imparted by ICH; or (v) is required to be disclosed by the Recipient to comply with the applicable laws or governmental regulations provided that the Recipient, where possible, notifies ICH of such requirement prior to any such disclosure.

5 Publication

5.1 The Recipient shall acknowledge ICH as the source of the Materials and for any other contribution (if any) in any publication which mentions the Materials.

5.2 The Recipient shall send the Supplier a copy of any proposed reports, publications or disclosures to third parties, whether oral or written, which describe work carried out using the Materials at least 45 days prior to any such disclosure or submission for publication. ICH shall have the right to delay publication or disclosure for up to 60 additional days to permit adequate steps to be taken to secure patent or other protection for the subject matter referred to therein and/or to require the deletion of any Confidential Information that would be disclosed by such report, publication or disclosure. ICH's rights under this clause shall not affect the Recipient's obligations under clause 4.

5.3 ICH shall be entitled to use all such reports, publications and disclosures.

6 Warranties & Liability

6.1 The Materials are experimental in nature and ICH makes no representation and gives no warranty or undertaking, in relation to them. As examples, but without limiting the foregoing, ICH gives no warranty: (i) that it owns all necessary property and other rights in the Materials and that their use will not infringe any patent, copyright, trade mark or other right owned by any third party; or (ii) that the Materials are of merchantable or satisfactory quality or fit for any particular purpose, have been developed with reasonable care and skill or tested, for the presence of pathogens or otherwise, or are viable, safe or non-toxic.

6.2 The Materials are made available by ICH free of charge as a service to the academic community and as such the Parties agree that the provisions of this clause 6.2 are reasonable. ICH shall have no liability to the Recipient, whether in contract, tort or otherwise, in relation to the supply of the Materials to the Recipient or their use or keeping

by the Recipient or by any other person, or the consequences of their use, to the maximum extent permitted under applicable law. The Recipient shall indemnify the Indemnified Parties from and against all Claims and Losses arising from such supply, use or keeping, including without limitation Claims and Losses arising from: (i) injury to the Recipient's employees and third parties; ; and (ii) use of the Materials within or outside the scope of this Agreement.

6.3 For the purposes of this Agreement: (i) 'Indemnified Parties' shall mean UCL, University College London and their respective directors, officers, employees, representatives and associated undertakings; (ii) 'Claims' shall mean all demands, claims, proceedings, penalties, fines and liability (whether criminal or civil, in contract, tort or otherwise); and (iii) 'Losses' shall mean all losses including without limitation financial losses, damages, legal costs and other expenses of any nature whatsoever.

6.4 For the avoidance of doubt ICH cannot guarantee that any Materials used or supplied under this Agreement are free from disease or infection (including, but not limited to, HIV, hepatitis B and TB) and the Recipient must treat all Materials as potentially infectious and hazardous.

6.5 The Recipient will ensure that its employees or others who on its behalf handle Materials are aware of the hazards and risks involved in handling Materials and of the provisions of this Agreement and, in particular clauses 6.1(ii) and 6.4. The Recipient shall ensure that it has in place all necessary safety procedures and practices and shall ensure that its employees and others comply with all safety requirements necessary for their well-being and that of others.

7 Duration and Termination

7.1 This Agreement shall come into force on the date on which it is signed by both Parties and shall remain in force for as long as the Recipient has ethical approval for the Research Programme up to a maximum period of 5 years (unless ICH agrees in writing to extend the term beyond such period), after which the Recipient shall dispose of the Materials as required by ICH in clause 7.4 When requesting any such extension, the Recipient shall provide ICH with proof of continuing ethical approval.

7.2 Either Party may terminate this Agreement immediately by notice in writing if:

(a) the other Party commits a material breach of this Agreement and, in the case of a breach capable of remedy, shall not have been remedied within 30 (thirty) days of the receipt by it of a notice identifying the breach and requiring its remedy; or

(b) the other Party becomes insolvent, or a petition of bankruptcy or any similar action under relevant bankruptcy or insolvency proceedings is filed by or against it, or a receiver is appointed with respect to any asset of the other Party or liquidation proceedings are commenced by or against it (except solvent and voluntary liquidation for reorganisation purpose).

7.3 ICH may additionally terminate this Agreement immediately without liability (i) if ICH is unable to supply the Materials, or any part of them, to the Recipient for any reason; (ii) if ICH is unable to permit the continued use of the Materials by the Recipient for any reason; or (iii) at any other time on request by ICH.

7.4 Upon termination of this Agreement the Recipient will immediately discontinue use of the Materials and will, upon the direction of ICH, either return to ICH or destroy all remaining Materials as agreed and in accordance with the Act and any relevant guidance issued by

the Human Tissue Authority or national equivalents. If the Materials are destroyed or exhausted, the Recipient shall provide ICH with prompt written confirmation of the same.

7.5 The obligations of the Parties under Clauses 1, 3, 4, 5, 6, 7.4, 7.5 and 8 of this Agreement shall survive termination of this Agreement, howsoever caused.

8 General

8.1 The Recipient shall not assign or transfer any interest, right, duty or obligation in this Agreement without the prior written agreement of ICH.

8.2 Except for the rights of the Indemnified Parties, a person who is not a party to this Agreement shall have no right under the Contracts (Rights of Third Parties) Act 1999 to enforce any term of this Agreement.

8.3 The Recipient shall ensure that the Principal Investigator and the Co-workers comply with the Recipient's obligations as if they were named as Parties to this Agreement.

8.4 The validity, construction and performance of this Agreement shall be interpreted in accordance with English law and the Parties hereby submit to the exclusive jurisdiction of the English Courts. Nothing in this clause shall however prevent either Party from seeking interim relief in any court of competent jurisdiction.

AGREED by the Parties through their authorised signatories:

For and on behalf of
ICH

Signed

CATRIN BALL
Print Name

INSTITUTE MANAGER
Title

11/9/2012
Date

For and on behalf of the
Recipient

Signed

GRAEME BROWN
Print Name

DIRECTOR TECHNOLOGY TRANSFER
Title

30/08/2012
Date

Acknowledged by the **Principal Investigator**

Signed

Helen Storr
Print Name

Dr
Title

28.08.2012
Date

Schedule A

Please specify the type and quantity of Materials that are required.

Human fetal adrenal (HFA) tissue from the HDBR collection: 6 PCW – 12 PCW (for immunohistochemistry and establishment of primary cell culture).
HFA from the FTB: 8-19 PCW (for immunohistochemistry).

HDBR collection: First trimester placental tissue (for establishment of primary cell culture, and as a positive control for experiments (both mRNA and protein expression of KISS1 and KISS1R have been shown to be high in first trimester placental trophoblast cells (Bilban et al. 2004)).

Studying tissue from 6PCW – 19 PCW inclusive will require 14 samples. Studies should ideally be performed in duplicate or triplicate. Fetal adrenal tissue will also be required to establish primary cell cultures. In total it is anticipated that a minimum of 40 samples over 18 months will be required for this project.

Schedule B

Description of the Research Programme including the purposes for which the Materials will be used.

Background
During pregnancy, the human fetal adrenal (HFA) cortex grows due to enlargement of the inner fetal zone (FZ). HFA growth is paralleled by increased steroidogenesis, which enables the feto-placental unit to maintain pregnancy.

Placental corticotropin-releasing hormone (CRH) and kisspeptin increase dramatically during pregnancy and their biological roles remain to be fully elucidated. Data from animal models suggest that sonic hedgehog (Shh) signalling plays an important role in adrenal development.

Laboratory studies
This project will be carried out in collaboration with John Achermann's group at ICH. The factors responsible for regulating growth and function of the HFA will be investigated. The spatio-temporal expression of the kisspeptin receptor (Kiss1R) together with CRHR receptor subtypes (CRHR1 and 2) will be examined in HFA tissue from a range of gestations, and the role of Shh signalling in HFA development will be clarified.

The tissue sections will be stained at ICH or at QMUL (under a MTA) using antibodies against Kiss1R and CRHR1 and 2, and co-stained with antibodies against markers of adrenocortical zones such as anti-CD56 (definitive zone (DZ)), or anti-SULT2A1 (FZ). Co-expression of Kiss1R and CRHR with Shh, a marker of subcapsular progenitor cells, will be investigated using serial *in situ* hybridization (for Shh) followed by immunofluorescence (for the receptors).

To obtain functional data on the regulation of the HFA, primary culture of HFA cells and placental cells will be established. . Isolation of DZ cells from FZ cells, based upon the expression of a cell surface marker of zonation, such as anti-CD56, will be achieved by discontinuous-gradient centrifugation and fluorescence-activated cell sorting (FACS). An alternative method will be to use laser capture microdissection (LCM) to isolate DZ/TZ cells from FZ cells. Subsets of HFA cells that are zone specific will then be cultured and after 24 hours when cells are no longer classed as relevant material under the Human Tissue Act, they will be transferred to QMUL under a MTA. The zone-specific cells will then be used in experiments to study the functional properties of each zone and actions and interactions of kisspeptin and CRH on each cell type. The expression of *KiSS1R* and *CRHR1* and *CRHR2* in subsets of cells by RT-PCR will be investigated, as well as the modification of receptor expression with exposure to kisspeptin and CRH. Kisspeptin stimulated steroid production will be investigated by mass spectral analysis of the cell media. Modification of steroid production with exposure to kisspeptin and CRH will also be studied.

APPENDIX 5: PARTICIPANT INFORMATION SHEET

Kisspeptin and CRH: Placental hormones in pregnancy that may influence premature birth and newborn adaptation to postnatal life

Patient information sheet

What is the purpose of this study?

The way in which normal pregnancy is maintained allowing an unborn baby to remain inside the mother until their due date is not well understood. Over the past 20 years the number of babies born prematurely, before their expected date of delivery, has risen. Premature birth is one of the biggest threats to unborn babies and is a major cause of sickness and death after birth, as well as long-term health problems.

During pregnancy the unborn baby communicates with the mother via the placenta, which connects the two together. Signals passing back and forth between mother and baby are likely to help maintain a pregnancy, enabling a baby to grow and develop. Hormones are natural chemical signals produced by the body, and we think that hormones produced by the placenta and by a baby's adrenal gland, (a small gland that sits on top of the kidneys) may be important in this process. This study is being carried out in order to better understand the way in which the placenta and the adrenal gland of the unborn baby communicate with each other via hormone signals. We would like to know if hormones produced by the placenta and the unborn baby's adrenal gland help to maintain pregnancy until the correct gestational due date as well as helping the baby grow and mature in preparation for life after birth.

It is already known that hormones produced by the unborn baby's adrenals signal to the placenta, affecting the way the placenta works as pregnancy progresses. We would like to know if a mutual

relationship exists: if hormones produced by the placenta can signal to the unborn baby's adrenal and affect the growth, development and the way this organ works during pregnancy, as well as progression of the pregnancy itself. Two hormones produced by the placenta are kisspeptin and corticotropin releasing hormone (CRH). The levels of both these hormones in the mother's blood increase dramatically as pregnancy progresses and it is not clear why this happens.

We would like to measure the levels of kisspeptin and CRH at different stages of pregnancy and see if these levels correlate with the growth of the unborn baby's adrenal glands during pregnancy, and outcomes of pregnancy including the gestation at which the baby is born, the production of steroid hormones from the baby's adrenal gland after birth and how well the baby adapts to life after birth.

Why have you been invited?

Previous scientific studies have looked at the effect of CRH on the progression of pregnancy, however many unanswered questions remain, as CRH levels do not always correlate with the onset of labour. The reason why kisspeptin levels increase so high as pregnancy progresses what kisspeptin does is unknown. There is no scientific data correlating levels of placental kisspeptin and CRH with growth of the unborn baby's adrenal gland and outcomes of pregnancy. We therefore aim to study women who have normal pregnancies booking here at the Royal London Hospital, and compare this group to women whose pregnancies are complicated by pre-eclampsia (a condition where the placenta does not work properly).

You have been invited because at your 12-week scan your Obstetric doctor noted a finding of abnormal uterine artery Doppler measurements. Previous studies carried out in this department have shown that this finding occurs in 1 in 5 of all pregnant women. This finding is associated with an increased risk of pre-eclampsia in 35% of cases. 65% of women, however, will go on to have an uncomplicated normal pregnancy. At this stage your Obstetric doctor will continue to follow you up closely. In conjunction with their follow up of your antenatal care we are inviting you to participate in our study, which is being run in collaboration with them.

How will the studies affect your treatment?

The study will not affect the antenatal care or treatment you are receiving. The results from the study may improve our understanding of factors that can influence pregnancy, which can be useful in the future.

What are the side effects of any treatment received when taking part?

There are no treatments included in this study. You will be required to attend additional appointments for extra ultrasound scans and blood tests however these will not harm your baby or you in any way.

Do you have to take part?

It is up to you to decide whether to take part or not because participation in the research study is completely voluntary. If you do agree to take part you will be given this information sheet to keep. In addition, you will also be given a consent form to sign. You are under no obligation to participate in any part of this study, and you may withdraw at any time without giving a reason. This will not affect the care you are receiving in any way.

What will happen if you take part?

You have been invited back for your second trimester anomaly ultrasound scan at 18–22 weeks. At this time if you give your consent to participate in this study you will be asked to sign a written consent form. You will have your anomaly scan performed in the Foetal Medicine Unit by a trained specialist senior obstetric doctor who will also perform a detailed scan to measure the size of the adrenal gland that sits on top of your baby's kidneys. This will not cause discomfort to you nor will it affect your baby in any way. The detailed scan will take around an extra 10 minutes in addition to your routine scan.

After the scan, you will be seen by a member of the research team who will take extra samples of blood (total ~10ml of blood) for the research study.

The detailed scans along with the blood tests will be repeated on 3 further occasions: at 28 weeks, 34 weeks, 38 weeks gestation. These additional scans and blood tests may require extra hospital visits and each appointment could last up to 30 minutes.

If your obstetric doctor wishes to follow you up more closely in the event of any complications, such as pre-eclampsia, the timing of these additional investigations will be adjusted to coincide with your follow up and minimise any inconvenience to you. You will be seen in the Foetal Medicine Unit at the Royal London Hospital by a doctor from the Obstetric team and a member of the research team on each occasion.

After delivery, a final blood test will be taken from you prior to discharge home. With your consent, at the time of delivery, we would also like to take a biopsy sample from the placenta, and some blood from the umbilical cord once the cord has been clamped and cut. These samples will be frozen and stored for future research. Any future research studies will be carried out only once ethical approval has been obtained.

About 5-7 days after the birth of your baby, we will invite you back to attend the hospital in order for your baby to have an abdominal ultrasound scan to again measure the adrenal gland. The scan will be performed by a specialist paediatric radiology consultant in the radiology department at the Royal London Hospital. This will not harm your baby or influence any medical care they may be receiving. Prior to discharge from hospital, you will have been provided with a urine bag and instructions on how to collect a urine sample from your baby. A member of the research team will take the urine sample from you after the scan. This sample will be used to measure hormone levels produced by your baby's adrenal gland. At this time we, with your consent, we would also like to take a whole blood sample (total ~2ml of blood) from your baby for storage. Your baby's Guthrie test can be performed by a trained senior paediatrician at the same time as the study blood sample is taken if you would like this to be done in hospital. Taking a blood sample can be uncomfortable for your baby but is done routinely at this age. The newborn screening test (Guthrie test), which is done at 5-7 days of life, usually by a midwife, is recommended for all babies. It tests babies for serious disorders for which early treatment can prevent mental retardation, physical disability, or death. The test is performed by pricking your baby's heel or taking blood from a vein to obtain 5 spots of blood on a special filter paper.

At 6-8 weeks we will invite you back again to attend the hospital in order for your baby to have a 2nd abdominal ultrasound scan and provide a urine sample.

If for whatever reason your baby is still in hospital, we will arrange for investigations to take place in the hospital depending on where your baby is.

Will my taking part in this study be kept confidential?

Only the medical team taking care of you and the individuals doing the research will have access to your personal information and the data collected during the study. If you consent to take part in the research the people conducting the study will abide by the Data Protection Act 1998, and the rights you have under this Act. You will be asked on the consent form whether you agree to your clinical information being shared with the research team. All the information that is collected about you during the course of the study will be kept strictly confidential. We will not share the clinical data or results with anyone other than the obstetric doctors involved in your care. All the clinical details and results will be kept on a secure, password protected computer database at Queen Mary University of London. This information will only be used for research purposes. We will notify your GP that you are taking part in the study unless you ask us not to.

What will be done with the information and samples used in the research?

All clinical information and samples that are collected during the course of this research will be used to gain further information about factors that may influence the timing of birth and the way a baby adapts to life after birth. Samples and the data will be stored securely. Only the study doctors will have access to your identity, the study data and study samples. The samples will be kept for future ethically approved research projects only if you consent for use of your stored sample for future research purposes. Your clinical data will be securely destroyed at the end of the research studies.

What if relevant new information becomes available?

If the study is stopped for any reason, we will tell you and arrange your continuing care with the relevant medical team. Likewise if any of the scans reveal any abnormalities, you will be referred to the appropriate team for further management as per routine. As there is limited information regarding normal levels of kisspeptin and CRH in pregnancy, and it is very unclear what these levels mean, this information will not be shared until completion of the study. Similarly growth of your unborn baby's adrenal gland will be monitored but again standardised normal measurements do not exist, so this

information will not be made available until completion of the study. The urine results from your baby will be shared with you and your GP if any abnormalities are detected and your baby will be referred to the appropriate team for further management.

What happens if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [see contact details below]. If however you would like to pursue this formally then please contact Patient Advice and Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint (telephone 020 359 42040 / 42050, or email PALS@bartsandthelondon.nhs.uk). You can also visit PALS in person at the Royal London Hospital.

What will happen to the results of this study?

The results of this research may be presented at scientific meetings and published in scientific journals. No identifiable patient information will be included and all data will be anonymised before presentation or publication.

Who is funding the research?

The Central and East London Comprehensive Local Research Network is currently meeting the cost of this study. No payment is being given to the doctors or researchers for including you in this study.

Who has reviewed the research?

The research proposal has been reviewed by a Local Ethics Committee, which has approved this study. The project is registered with the Research and Development Office at Barts Health NHS Trust.

Who can I contact to find out more about this study?

If you have any questions please contact the following research team members:

Dr Harshini Katugampola, Dr Muriel Meso (Tel: 020 78826243 E-mail: endocrinology@qmul.ac.uk)

Thank you for considering taking part in this important study. We very much appreciate your help.

APPENDIX 6: CONSENT FORM

Barts and The London School of Medicine and Dentistry

Barts Health NHS NHS Trust

PATIENT CONSENT FORM

Kisspeptin and CRH: Placental hormones in pregnancy that may influence premature birth and newborn adaptation to postnatal life

Investigators: Dr Harshini Katugampola, Dr Muriel Meso
Tel: 020 78826243 **Fax:** 020 7882 6197 **E-mail:** endocrinology@qmul.ac.uk

Please initial box if you agree

1.	I confirm that I have read and understand the information sheet dated 1 July 2012 (version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or my legal rights being affected.	
3.	I agree to my clinical information being shared with the research team. This includes relevant clinical details and the results of biochemical tests and scans that have been performed.	
4.	I agree to allow my medical information and that of my baby to be entered on a secure, confidential computer database.	
6.	I agree to provide samples of blood as specified in the information sheet dated 1 July 2012 (version 1). I agree to the research studies being undertaken on the sample. I understand that any results arising from this research work will be kept strictly confidential.	
6.	I agree to have abdominal ultrasound scans as detailed in the information sheet dated 1 July 2012 (version 1).	
7.	I agree to provide samples of my baby's urine for analysis as specified in the information sheet dated 1 July 2012 (version 1). I agree to research studies being undertaken on the sample. I understand that any results arising from this research work will be kept strictly confidential.	
8.	I agree for my baby to have abdominal ultrasound scans as detailed in information sheet dated 1 July 2012 (version 1).	
9.	I agree to provide a sample of my baby's blood for storage for future research studies. Any future studies will have Ethics Committee permission.	
10.	I agree to a biopsy of my placenta and cord blood samples being taken after delivery and stored for future research studies. Any future studies will have Ethics Committee permission.	
11.	I agree to my GP being informed of my participation in the study.	
12.	Following the present research project, any residual (left-over) blood/urine samples may be stored and used by the research team at Barts and the London School of Medicine and Dentistry (QMUL) for future research studies. Any future studies will have Ethics Committee permission. Your clinical data will be securely destroyed at the end of the research studies. All staff undertaking future studies will abide by the Data Protection Act 1998 with any medical information relating to your child being kept confidential.	

.....	.....	.....
Name of parent/carer	Date	Signature
.....	.....	.....
Name of person taking consent	Date	Signature
.....	.....	.....
Investigator	Date	Signature

Version 1 1st July 2012

APPENDIX 7: GP LETTER

Barts and The London School of Medicine and Dentistry

Barts Health NHS NHS Trust

Dear Dr

This is to inform you that your patient ..... DOB ..... NHS number ..... has consented to take part in a research study entitled *'Kisspeptin and CRH: Placental hormones in pregnancy that may influence premature birth and new born adaptation to postnatal life'*. This involves serial antenatal ultrasound scans at the Royal London Hospital and maternal serological tests during pregnancy and post-partum.

The infants of mothers enrolled in this study will have a postnatal adrenal ultrasound, a blood test and a collection of urine for steroid analysis at 5-7 days of life. At the time of the blood test, the Guthrie test will also be performed in the hospital if the parents would like this to be done. The postnatal ultrasound and urine sample will be repeated at 6-8 weeks.

We will inform you of any results requiring follow up. Please do not hesitate to get in touch with us if further information is required (contact below).

Yours Sincerely

Dr Harshini Katugampola and Dr Muriel Meso

Clinical Research Fellows in Paediatric Endocrinology

Centre for Endocrinology, Charterhouse Square, London EC1M 6BQ

Tel: 020 7882 6243
Fax: 020 7882 6197

E-mail: endocrinology@qmul.ac.uk

Version 1 **1July 2012**

APPENDIX 8. Participant demographics, gestation, foetal adrenal volume and kisspeptin levels at each antenatal visit.

				Visit 1			Visit 2			Visit 3			Visit 4		
PN	Age	Ethnicity	Gravidity/ Parity	GA (wks)	FA vol (cm^3)	KP (pmol/)	GA (wks)	FA Vol (cm^3)	KP (pmol/)	GA (wks)	FA Vol (cm^3)	KP (pmol/)	GA (wks)	FA Vol (cm^3)	KP (pmol/)
1	24	O	G1, P0	19.43	0.34	2938.2	26.43	1.45	3680.5	-	-	5159.5	37.71	3.27	4997.9
2	29	WE	G1, P0	-	-	2525.1	27.00	0.16	2524.2	33.00	1.17	4284.0	38.00	8.64	2210.0
3	34	FEA	G1, P0	20.14	1.23	5719.2	27.14	3.18	5957.4	33.57	4.84	6686.0	37.57	0.82	5515.9
4	28	AC	G4, P1	20.71	0.82	2131.5	27.71	1.16	5301.0	36.00	2.41	5092.3	39.71	13.61	3968.5
5	29	SEA	G2, P1	20.43	0.48	2981.7	27.29	4.94	3130.9	34.29	0.57	6164.7	38.29	1.38	3338.8
6	28	WE	G3, P1	20.29	1.32	2383.4	28.86	0.21	3718.4	34.00	9.11	4924.3	37.86	7.95	3970.8
7	32	WE	G1, P0	19.29	0.07	2367.2	29.29	1.21	5615.0	34.71	1.03	6235.0	38.71	7.87	3702.8
8	34	WE	G1, P0	20.57	0.15	4355.9	28.43	1.32	6381.8	34.57	4.45	7430.5	38.00	4.17	7774.3
9	30	AC	G3, P1	20.43	0.10	1679.5	28.29	0.24	3273.3	-	-	2992.9	38.29	1.67	2883.1
10	20	AC	G1, P0	20.00	0.55	3470.0	27.71	2.83	6500.8	33.71	2.08	6805.1	37.14	2.16	5967.5
11	35	WE	G1, P0	22.43	0.15	2979.1	28.29	2.76	6574.4	34.43	4.81	6890.9	37.71	9.60	4818.6

				Visit 1			Visit 2			Visit 3			Visit 4		
PN	**Age**	**Ethnicity**	**Gravidity/ Parity**	**GA (wks)**	**FA vol (cm^3)**	**KP (pmol/)**	**GA (wks)**	**FA Vol (cm^3)**	**KP (pmol/)**	**GA (wks)**	**FA Vol (cm^3)**	**KP (pmol/)**	**GA (wks)**	**FA Vol (cm^3)**	**KP (pmol/)**
12	30	SEA	G1, P1	21.00	0.12	3885.0	29.86	2.90	2252.6	35.00	1.92	5962.4	37.43	1.17	3619.7
13	25	SEA	G2, P0	20.14	0.08	1974.6	28.00	0.24	1428.8	34.00	1.50	3235.8	38.00	8.60	2107.7
14	31	SEA	G2, P1	-	-	3370.5	29.14	2.80	5278.9	36.14	9.50	915.9	-	-	-
15	37	WE	G1, P0	20.14	0.08	2933.6	27.71	3.15	4217.3	33.71	2.37	4159.6	37.86	5.73	4916.2
16	29	SEA	G3, P2	20.43	0.43	1850.8	28.57	1.13	3965.1	34.71	1.71	4805.9	38.86	2.16	1977.3
17	32	FEA	G3, P1	20.14	0.23	1541.0	26.29	0.36	1976.4	34.43	2.29	2506.1	38.29	2.70	3718.1
18	31	M	G1, P0	20.14	0.49	3437.8									
19	31	SEA	G1, P0	20.00	0.08	3254.3	28.00	0.71	3918.9	34.14	1.34	3998.8	38.00	1.65	2613.1
20	25	M	G1. P0	20.00	0.88	3648.5	27.71	1.56	5721.3	34.71	4.36	6649.5	37.86	0.75	5566.2
21	31	WE	G1, P0	20.29	0.35	2949.1	28.29	0.45	2823.1						
22	28	SEA	G4, P2	20.43	0.19	1624.2	28.43	0.44	1098.3	34.43	0.95	1396.8	-	-	-
23	29	WE	G2, P0	22.57	0.41	1781.0	27.71	1.4	4484.9	33.71	1.71	-			

				Visit 1			Visit 2			Visit 3			Visit 4		
PN	**Age**	**Ethnicity**	**Gravidity/ Parity**	**GA (wks)**	**FA vol (cm^3)**	**KP (pmol/)**	**GA (wks)**	**FA Vol (cm^3)**	**KP (pmol/)**	**GA (wks)**	**FA Vol (cm^3)**	**KP (pmol/)**	**GA (wks)**	**FA Vol (cm^3)**	**KP (pmol/)**
24	32	M	G2, P1	20.43	0.13	2822.2	28.43	0.42	3666.8	34.29	1.06	5903.8	37.29	1.44	4683.6
25	24	WE	G1, P0	19.57	0.10	3405.2	28.71	0.29	6034.0	35.29	0.31	6696.1	38.29	0.78	5560.9
26	30	BA	G3, P2	20.43	0.12	2017.6	28.43	0.32	-	34.63	-	1678.7	37.71	0.88	2942.8
27	25	WE	G1, P0	20.14	0.20	1542.9	28.29	0.59	2081.8	34.86	0.44	4098.7	39.29	2.36	3779.0
28	17	SEA	G1, P0	19.86	0.01	3207.7	27.57	0.10	3358.0	34.71	0.31	3460.7	38.71	0.33	1694.6
29	17	FEA	G2, P1	19.86	0.04	3276.0	27.86	0.33	5476.7	35.71	0.94	5520.2	39.00	-	-
30	17	SEA	G2, P0	20.71	1.17	1793.8	28.57	0.25	3952.7	33.43	0.80	2568.6			
31	17	BA	G2, P0	20.14	0.21	2507.7	27.86	0.06	4959.1	34.00	0.84	3812.6	38.43	-	2345.8
32	17	FEA	G1, P0	20.57	0.02	2403.9	28.14	0.33	5687.2	34.43	1.55	4103.6	39.71	-	3218.0
33	17	SEA	G2, P0	20.14	0.07	882.0	28.14	0.10	2169.7	33.71	1.04	3019.1	41.14	-	2051.3

PN, participant number; -, data missing (subjects failed to attend for scans / blood test); we, white European; fea, Far East Asian; sea, south east Asian; bc, black Caribbean; ba, black African; m, mixed race; o, other; GA, gestational age (weeks). Subject numbers 21 and 23 moved area before completion of the study (after visits 2 and 3, respectively). Subjects 18 and 30 had premature delivery at 26 and 33 weeks gestation, respectively.

APPENDIX 9. Neonatal participant demographics, gestation, mode of delivery, adrenal volume at each postnatal visit, intra- or post-partum complications

					Visit 1		Visit 2		Visit 3		
PN	**Gestation (weeks)**	**Birth weight (g)**	**Mode of delivery**	**Sex**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Complications**
1	40.71	3000	Em LSCS	F	-	-	5	0.07	-	-	PROM
2	42.14	3680	Forceps	F							None
3	39.43	2610	SVD	F							None
4	41.29	3230	Em LSCS	F	1	0.11	-	-	35	0.04	MEC, NNU respiratory support
5	39.14	2980	SVD	F	-	-	-	-	42	0.02	None
6	39.29	2420	IOL VD	M							Pre-eclampsia, SGA
7	39.00	3200	SVD	M							None
8	40.86	3640	SVD	M	-	-	-	-	44	0.04	Sepsis, NNU

					Visit 1		Visit 2		Visit 3		
PN	**Gestation (weeks)**	**Birth weight (g)**	**Mode of delivery**	**Sex**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Complications**
9	39.29	2680	Em LSCS	F	-	-	5	0.12	41	0.04	Pre-eclampsia
10	40.00	3220	SVD	M	-	-	6	0.14	-	-	None
11	40.14	3250	SVD	F							None
12	38.29	3040	Em LSCS	M	2	0.08			49	0.07	Placental abruption
13	40.86	3000	SVD	M	1	0.23	5	0.11	38	0.05	None
14	39.29	3120	SVD	F	-	-	7	0.06	-	-	None
15	40.57	3280	Ventouse	M							None
16	41.71	3120	IOL VD	M	-	-	7	0.16	45	0.03	None
17	39.57	3515	Forceps	M	-	-	-	-	44	0.05	Sepsis, NNU

					Visit 1		Visit 2		Visit 3		
PN	**Gestation (weeks)**	**Birth weight (g)**	**Mode of delivery**	**Sex**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Complications**
18	26.86	950	SVD	M							Preterm,NNU, respiratory support, sepsis
19	39.14	3360	El LSCS	F	-	-	-	-	40	0.009	None
20	41.00	3620	SVD	M							None
21	37.14	1720	IOL VD	M	1	0.3	5	0.22	44	0.034	Pre-eclampsia, SGA, NNU
22	39.86	3500	SVD	M	1	0.16	5	0.08	-	-	None
23	39.29	3540	El LSCS	F							None
24	39.29	3040	IOL, VD	F	1	0.37	-	-	47	0.04	None
25	42.00	3640	SVD	F	2	0.07	5	0.12	48	0.02	MEC

					Visit 1		Visit 2		Visit 3		
PN	**Gestation (weeks)**	**Birth weight (g)**	**Mode of delivery**	**Sex**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Age (day)**	**Adrenal Volume (cm^3)**	**Complications**
26	41.14	3580	SVD	M							polycythaemia neonatorum, partial dilutional exchange, ?sepsis
27	39.00	2900	El LSCS	M	-	-	7	0.04	-	-	None
28	33.57	1340	EM LSCS	M	-	-	-	-	43	0.02	?sepsis, NNU
29	38.43	2640	SVD	M	-	-	5	0.07	-	-	None
30	39.71	3060	Ventouse	M	2	0.05	-	-	-	-	None
31	41.14	3300	SVD	M	-	-	-	-	46	0.04	None

PN, participant number; -, data missing (subjects failed to attend for scans); F, female; M, male; IOL, induction of labour; Em LSCS, emergency c-section; El, elective c-section; SVD, spontaneous vaginal delivery ; PROM, prolonged rupture of membranes; MEC, meconium stained liquor; NNU, neonatal unit admission; SGA, small for gestational age. Parental consent for ten neonatal participants (2, 3, 6, 7, 11, 15, 18, 20, 23, 26) was not obtained for postnatal scans.

APPENDIX 10. Maternal serum biomarker measurements of placental sufficiency at each antenatal visit

	Visit 1			Visit 2			Visit 3			Visit 4		
PN	**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**	**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**	**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**	**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**
1	19.43	41723	304.6	26.43	21424	899.1	-	-	666.3	37.71	3.27	651
2	-	-	-	27.00	-	-	33.00	-	-	38.00	-	-
3	20.14	40525	151.5	27.14	36893	-	33.57	-	-	37.57	-	102
4	20.71	12554	-	27.71	20947	-	36.00	-	-	39.71	-	-
5	20.43	21476	243.5	27.29	21996	420.1	34.29	-	408	38.29	-	133.2
6	20.29	20776	251	28.86	7321	325.8	34.00		-	37.86	-	50.61
7	19.29	16165	-	29.29	-	-	34.71	-	367.8	38.71	-	227.8
8	20.57	4423	264.8	28.43	4278	-	34.57	-	412.5	38.00	-	288.7
9	20.43	29984	572.2	28.29	31368	1090	-	-	415.4	38.29	-	234
10	20.00	9158	215.3	27.71	4722	544.2	33.71	-	757.9	37.14	-	143.6
11	22.43	19946	-	28.29	-	-	34.43	-	-	37.71	-	-

		Visit 1			Visit 2			Visit 3			Visit 4		
PN		GA (wks)	hCG (U/L)	PlGF (pg/ml)	GA (wks)	hCG (U/L)	PlGF (pg/ml)	GA (wks)	hCG (U/L)	PlGF (pg/ml)	GA (wks)	hCG (U/L)	PlGF (pg/ml)
12		21.00	25442	323.3	29.86	-	519.1	35.00	-	465.7	37.43	-	302.8
13		20.14	24602	145.6	28.00	-	313.8	34.00	-	385.7	38.00	-	167.5
14		-	-	-	29.14	-	-	36.14	-	-	-	-	-
15		20.14	30758	-	27.71	-	-	33.71	-	-	37.86	-	-
16		20.43	12452	-	28.57	-	1152	34.71	-	2200	38.86	-	816.2
17		20.14	32410	353.3	26.29	-	494.6	34.43	-	308.2	38.29	-	282.4
18		20.14	17801	293.3									
19		20.00	23176	246.7	28.00	-	473.9	34.14	-	801.8	38.00	-	530.8
20		20.00	17282	162.8	27.71	-	665.1	34.71	-	361.7	37.86	-	250.8
21		20.29	12366	1351	28.29	-	278.1						
22		20.43	6892	172.8	28.43	-	143.4	34.43	-	76.17			
23		22.57	47460	298.9	27.71	-	-	33.71	-	-	37.29	-	-

		Visit 1			Visit 2			Visit 3			Visit 4		
PN		**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**	**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**	**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**	**GA (wks)**	**hCG (U/L)**	**PlGF (pg/ml)**
24		20.43	35217	224.8	28.43	-	878.6	34.29	-	1018	38.29	-	515.1
25		19.57	25689	323.3	28.71	-	619.1	35.29	-	465.7	37.71	-	302.8
26		20.43	6379	-	28.43	-	-	34.63	-	-	39.29	-	-
27		20.14	41814	-	28.29	-	-	34.86	-	-	38.71	-	-
28		19.86	20206	100.7	27.57	-	443.9	34.71	-	136.3	39.00	-	-
29		19.86	15811	48.09	27.86	-	24.05	35.71	-	20.47	37.29	-	-
30		20.71	14921	261	28.57	-	890.4	33.43	-	422.7			
31		20.14	15608	-	27.86	-	-	34.00	-	-	38.43	-	-
32		20.57	8362	-	28.14	-	-	34.43	-	-	39.71	-	-
33		20.14	-	-	28.14	-	-	33.71	-	-	41.14	-	-

PN, participant number; -, data missing (subjects failed to attend for scans). GA, gestational age (weeks). Subject numbers 21 and 23 moved area before completion of the study (after visits 2 and 3, respectively). Subjects 18 and 30 had premature delivery at 26 and 33 weeks gestation, respectively. Subjects 6, 9, and 22 developed pre-eclampsia (highlighted yellow.